SECTION VIII. VIRUS MONITORING PROTOCOL FOR THE ICR

TABLE OF CONTENTS

FOREWORD

The surface water treatment rule (40 CFR Part 141) established the maximum contamination level for enteric virus in public water systems by requiring that systems using surface water or ground water under the influence of surface water reduce the amount of virus in source water by 99.99%. The rule requirements are currently met on basis of treatment alone (e.g., disinfection and/or filtration), and thus the degree of actual protection against waterborne viral disease depends upon the source water quality. Utilities using virus-free source water or source water with low virus levels may be overtreating their water, while utilities using highly contaminated water may not be providing adequate protection. To determine more adequately the level of protection from virus infection and to reduce the levels of disinfection and disinfection byproducts, where appropriate, the U.S. EPA is requiring all utilities serving a population of over 100,000 to monitor their source water for viruses monthly for a period of 18 months. Systems finding greater than one infectious enteric virus particle per liter of source water must also monitor their finished water on a monthly basis. The authority for this requirement is Section 1445(a)(1) of the Safe Drinking Water Act, as amended in 1986.

This Virus Monitoring Protocol was developed by virologists at the U.S. EPA and modified to reflect consensus agreements from the scientific community and comments to the draft rule. The procedures contained herein do not preclude the use of additional tests for research purposes (e.g., polymerase chain reaction-based detection methods for non-cytopathic viruses).

The concentrated water samples to be monitored may contain pathogenic human enteric viruses. Laboratories performing virus analyses are responsible for establishing an adequate safety plan and must rigorously follow the guidelines on sterilization and aseptic techniques given in **Part 5**.

Analytical Reagent or ACS grade chemicals (unless specified otherwise) and deionized or distilled reagent grade water (dH_2O; see Table IV-1) should be used to prepare all media and reagents. The dH_2O must have a resistance of greater than 0.5 megohms-cm at 25°C, but water with a resistance of 18 megohms-cm is preferred. Water and other reagent solutions may be available commercially. For any given section of this protocol only apparatus, materials, media and reagents that are not described in previous sections are listed, except where deemed necessary. The amount of media prepared for each Part of the Protocol may be increased proportionally to the number of samples to be analyzed.

PART 1 — SAMPLE COLLECTION PROCEDURE

APPARATUS AND MATERIALS

Several configurations are given below for the assembly of the filter apparatus. The standard filter apparatus will be used for all sampling, except where a prefilter, dechlorination or pH adjustment are required.

1. Standard filter apparatus (see **Figure VIII-1**).

   a. Parts needed (letters in bold print represent the origin of the abbreviations used to identify parts in the figures):

      i. One BR — **B**ackflow **R**egulator (Watts Regulator[1] Product Series 8 — ¾" Hose Connection Vacuum Breaker).

      ii. One SF — **S**wivel **F**emale insert with garden hose threads (United States Plastic Product No. 63003).

      iii. Three sections of BT — **B**raided **T**ubing, ½" clear (Cole-Parmer Product No. G-06401-03).

      iv. Six HC1 — **H**ose **C**lamps (Cole-Parmer Product No. G-06403-20).

      v. One HF1 — **H**ose **F**itting, nylon, ⅜" male NPT × ½" tubing ID (United States Plastic Product No. 61141).

      vi. One PR — **P**ressure **R**egulator (Watts Regulator Product No. ⅜" 26A (or 263A), Suffix B).

      vii. One PN — **P**VC **N**ipple, ⅜" male NPT (Ryan Herco Product No. 3861-057; not required with the 263A regulator).

      viii. One TE — PVC **TE**E with ⅜" female NPT ports (Ryan Herco Product No. 3805-003; not required with the 263A regulator).

      ix. One RB1 — **R**educing **B**ushing, ⅜" NPT(M) × ¼" NPT(F) (Cole-Parmer Product No. G-06349-32; not required with the 263A regulator).

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[1]See **Part 7** for addresses of the vendors listed. The vendors listed in this protocol represent one possible source for required products. Other vendors may supply the same or equivalent products.

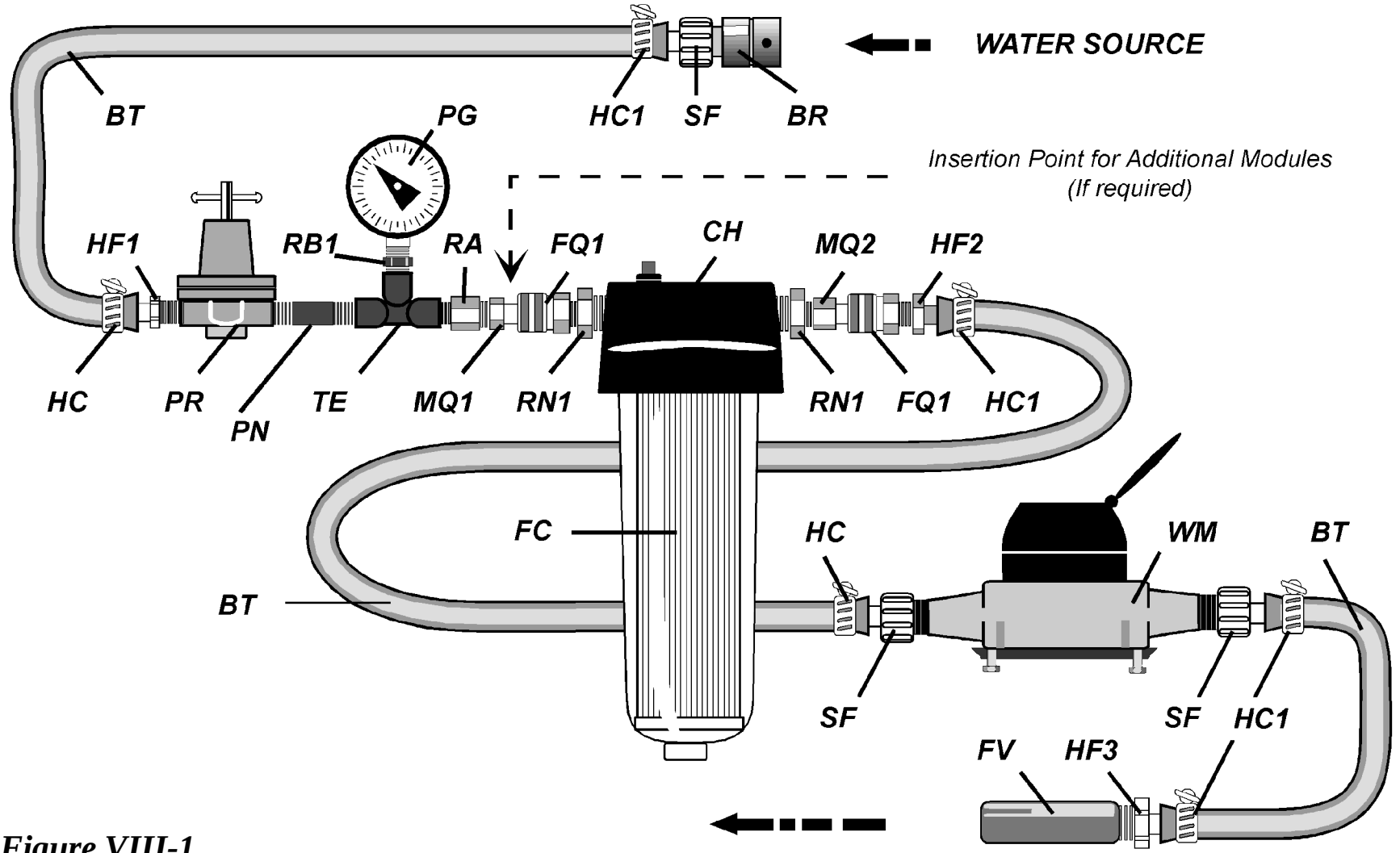


Figure VIII-1.
Standard Filter Apparatus

x. One PG — **P**ressure **G**auge 0-30 pound per square inch (PSI; Cole-Parmer Product No. G-68004-03; place in ¼" gauge port if using the 263A regulator).

xi. One RA — **R**educing **A**daptor, ½" female NPT × ⅜" male NPT (Cincinnati Valve and Fitting Product No. SS-8-RA-6).

xii. One MQ1 — **M**ale **Q**uick Connect, ½" male NPT (Cincinnati Valve and Fitting Product No. SS-QF8-S-8PM; appropriate hose fittings and braided tubing can be substituted for quick connects).

xiii. Two FQ1 — **F**emale **Q**uick Connects, ½" female NPT (Cincinnati Valve and Fitting Product No. SS-QF8-B-8PF).

xiv. Two RN1 — **R**educing **N**ipples, ¾" male NPT × ½" male NPT (Cole-Parmer Product No. G-06349-35).

xv. One CH — **C**artridge **H**ousing with wench (Cuno Product No. AP11T).

xvi. One FC — **F**ilter **C**artridge, positively charged 1MDS, ZetaPor Virosorb (Cuno Product No. 45144-01-1MDS).

xvii. One MQ2 — **M**ale **Q**uick Connect, ½" female NPT (Cincinnati Valve and Fitting Product No. SS-QF8-S-8PF).

xviii. One HF2 — **H**ose **F**itting, ½" male NPT × ½"tubing ID (United States Plastic Product No. 62142).

xix. One WM — **W**ater **M**eter (Neptune Equipment Product No. ⅝" Trident 10). The water meter should be used in a horizontal position and protected from freezing. The order should specify that the meters be rated in gallons (1 gal = 0.1337 ft^3 or 3.7854 L). If not specified, meters may be rated in cubic feet (1 ft^3 = 7.481 gal or 28.316 L).

xx. One HF3 — **H**ose **F**itting, nylon, ¾" male NPT × ½" tubing ID (United States Plastic Product No. 61143).

xxi. One FV — **F**low Control **V**alve (Plast-O-Matic Valves Product No. FC075B-3-PVC).

b. Apparatus assembly — the standard filter apparatus consists of three modules: the regulator module, the cartridge housing module and the discharge module.

Teflon tape (Cole-Parmer Product No. G-08782-27) must be used on all threaded, non-compression fittings. It is recommended that apparatus assembly be performed by the analytical laboratory contracted by the utility to analyze ICR samples for viruses).

i. Regulator module — in order, as shown in **Figure VIII-1**, connect the backflow regulator (BR) to a swivel female insert (SF). Clamp a piece of braided tubing (BT) onto the tubing connector of the swivel female insert using a hose clamp (HC1). Clamp the other end of the tubing to a ⅜ × ½" hose fitting (HF1). Screw the fitting into the inlet of the pressure regulator (PR). Connect the outlet of the pressure regulator to the PVC TEE (TE) via a PVC nipple (PN). Connect the pressure gauge (PG) to the top of the PVC TEE using the reducing bushing (RB). Attach a reducing adaptor (RA) to the remaining connection on the PVC TEE. Add a male quick connect (MQ1) to the reducing adaptor.

ii. Cartridge housing module — Attach a female quick connect (FQ1) to a reducing nipple (RN1). Connect the reducing nipple to the inlet of the cartridge housing (CH). Attach another reducing nipple to the outlet of the housing. Attach a male quick connect (MQ2) to the reducing adaptor.

iii. Discharge module — attach a female quick connect (FQ1) to a hose fitting (HF2). Connect a piece of braided tubing to the hose fitting with a hose clamp (HC1). Clamp the other end of the braided tubing to a swivel female insert with another hose clamp. Attach a swivel female insert to the inlet of the water meter (WM). Attach another swivel female insert to the outlet of the meter and connect a piece of braided tubing with a hose clamp. Clamp the other end of the tubing to a hose fitting (HF3) with a hose clamp. Screw the fitting into the inlet of the flow control valve (FV). An additional hose fitting (not shown) may be added to the flow control valve for the attachment of a sufficient length of tubing to reach a drain. The discharge module does not have to be sterilized.

iv. Connect the cartridge housing module to the regulator module at the quick connect. The combined regulator and cartridge housing modules should be sterilized with chlorine as described in **Part 5**. Presterilize a 1MDS filter cartridge (FC) as described in **Part 5** and place it into the cartridge housing using aseptic technique. Replace the housing head of the cartridge housing and tighten with a cartridge housing wench. Check to ensure that the filter is adequately sealed by shaking the housing. Adequately sealed filters should not move. For convenience during shipping, the regulator and cartridge housing modules may be separated. Seal all openings into the modules with sterile aluminum foil.

2. Prefilter module for waters exceeding 75 nephelometric turbidity units (NTU) and for any other conditions that prevent the minimum sampling volumes from being obtained (see **Figure VIII-2**).

a. Additional parts needed: One PC — 10 µm Polypropylene **P**refilter **C**artridge (Parker Hannifin Product No. M19R10-A); in addition, a female quick connect (FQ1), two reducing nipples (RN1), a cartridge housing (CH) and a male quick connect (MQ2) as described for the standard apparatus are needed.

b. Module assembly — in order, as shown for the prefilter module in **Figure VIII-2**, attach a female quick connect (FQ1) to a reducing nipple (RN1). Connect the reducing nipple to the inlet of the cartridge housing (CH). Attach another reducing nipple to the outlet of the housing. Attach a male quick connect (MQ2) to the reducing adaptor. Sterilize the unit with chlorine as described in **Part 5** and add a presterilized polypropylene prefilter cartridge using aseptic technique. Cover the ends with sterile aluminum foil. The prefilter module may be sent to the utility and stored in a clean location until needed.

3. Injector modules for source or finished water requiring pH reduction and for finished waters requiring dechlorination (see **Figure VIII-2**).

a. Additional parts needed:

i. Two FQ2 — **F**emale **Q**uick Connects, ½" male NPT (Cincinnati Valve and Fitting Product No. SS-QF8-B-8PM).

ii. Four ME — **M**ale **E**lbows, ⅜" male NPT (Cincinnati Valve and Fitting Product No. SS-6-ME).

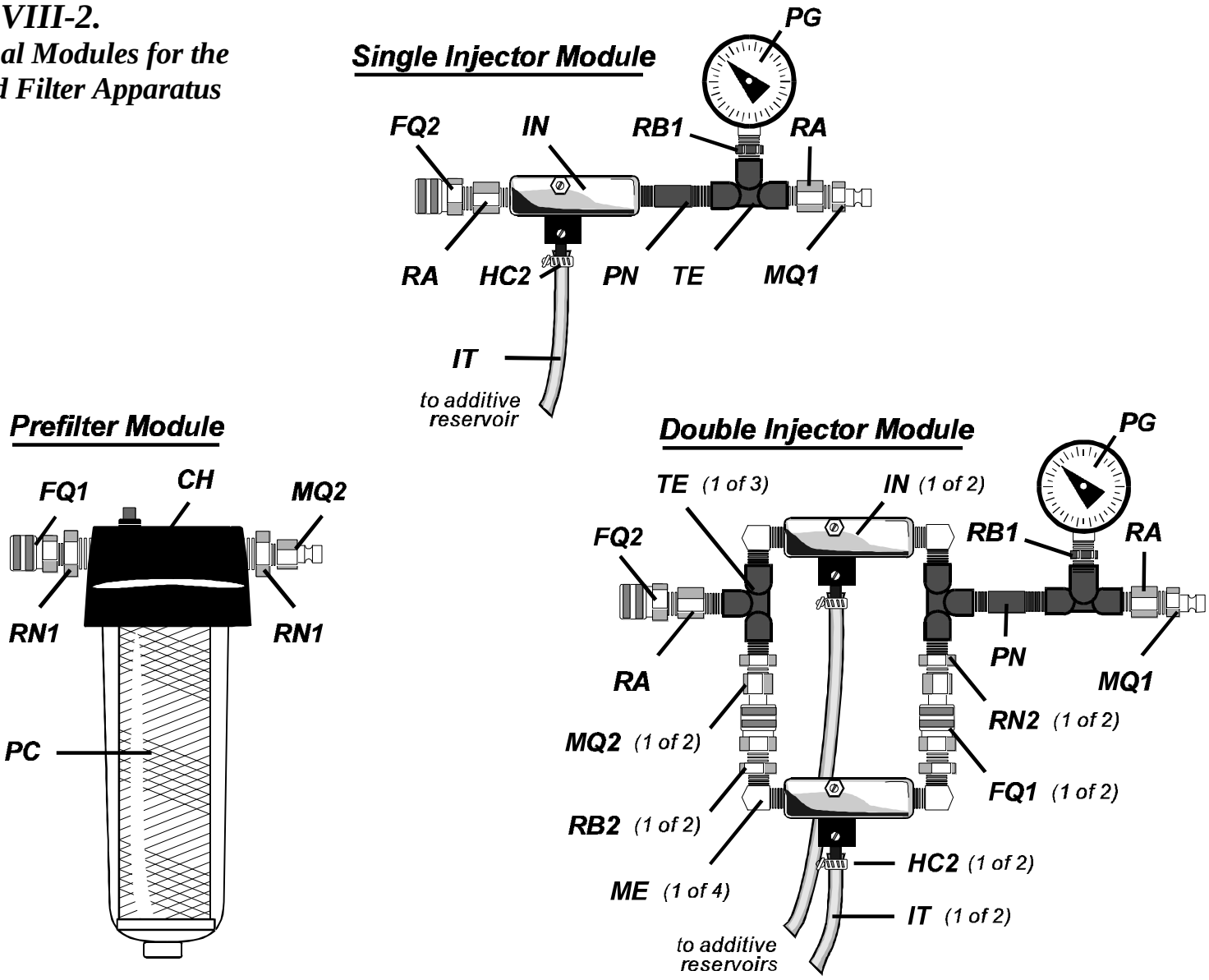
iii. Two RN2 — **R**educing **N**ipples, ⅜" male NPT × ½" male NPT (Cole-Parmer Product No. G-6349-85).

iv. Two RB2 — **R**educing **B**ushings, ⅜" female NPT × ½" male NPT (Cole-Parmer Product No. G-06349-34).

v. Three IN — In-line **IN**jectors (DEMA Engineering Product No. 203B ⅜" female NPT; a metering pump and appropriate connectors may be substituted for an injector).

vi. Three HC2 — **H**ose **C**lamps (Cole-Parmer Product No. G-06403-10).

Figure VIII-2.
Additional Modules for the Standard Filter Apparatus



vii. In addition, four reducing adaptors (RA), four PVC TEEs (TE), two PVC nipples (PN), two reducing bushings (RB1), two pressure gauges (PG), two female quick connects (FQ1), two male quick connects (MQ1) and two male quick connects (MQ2) as described for the standard apparatus are needed. Two union ball joints, ⅜" female NPT (not shown; Cincinnati Valve and Fitting Product No. SS-6-UBJ) and two PVC nipples may be used in place of the two reducing nipples (RN2), male quick connects (MQ2), female quick connects (FQ1) and reducing bushings (RB2) used with the double injector module.

b. Module assembly:

i. Single Injector Module — assemble the parts in order as shown for the single injector module in **Figure VIII-2**. Attach a female quick connect (FQ2) to a reducing adaptor (RA). Connect the adaptor to the inlet of the injector (IN). Connect the outlet of the injector to a PVC TEE (TE) via a PVC nipple (PN). Connect a pressure gauge (PG) to the top of the PVC TEE using a reducing bushing (RB1). Attach a reducing adaptor (RA) to the remaining connection on the PVC TEE. Add a male quick connect (MQ1) to the reducing adaptor.

ii. Double Injector Module — assemble the parts as shown for the double injector module in **Figure VIII-2**. Assemble the main portion by attaching a female quick connect (FQ2) to a reducing adaptor (RA). Connect the adaptor to the top connector of a PVC TEE (TE). Add a male elbow (ME) to one of the connections on the PVC TEE. Attach a reducing nipple (RN2) to the other connection. If using a union ball joint in place of the quick connects, attach a PVC nipple (not shown) to the other connection. Add a male quick connect (MQ2) to the reducing nipple or add one portion of a union ball joint (not shown) to the PVC nipple. Connect the inlet side of an injector (IN) to the male elbow. Attach another male elbow to the outlet of the injector. Connect the male elbow to another PVC TEE. Connect a reducing nipple (RN2 or PVC nipple) to the other end of the second PVC TEE. Add a male quick connect (MQ2) to the reducing nipple as above (or add one portion of the second union ball joint to the PVC nipple). Connect the top connector of the second PVC TEE to a third PVC TEE via a PVC nipple (PN). Connect a pressure gauge (PG) to the top of the third PVC TEE using a reducing bushing (RB1). Attach a reducing adaptor (RA) to the remaining connection on the third PVC TEE. Add a male quick connect (MQ1) to the reducing adaptor. Attach two male elbows (ME) to the inlet and outlet of a second injector (IN). Connect two reducing bushings (RB2) or, if used, the bottom portion or the two union ball joints (not shown) to the male elbows. Connect a female quick connect (FQ1) to each reducing bushing. Orient the second injector so that the direction of flow is the same as the first injector (the arrows on the injectors should both point towards the pressure gauge side of the assembly). Connect the two female quick connects to the male

quick connects of the main portion to complete the assembly or, if used, connect the two portions of the union ball joints.

iii. Sterilize the single and double modules with chlorine as described in **Part 5**. Cover the ends, including the injector port, with sterile aluminum foil. Sterilize the inside and outside surfaces of the **I**njector **T**ubing (IT; injector tubing is supplied with each injector). Place the tubing in a sterile bag or wrapping in such a way that the ends may be removed without contaminating them. The injector modules may be shipped to the utility and stored in a clean location until needed.

4. Portable pH probe (Omega Product No. PHH-1X)

5. Portable temperature probe (Omega Product No. HH110).

6. Commercial ice packs (Cole-Parmer Product No. L-06346-85).

7. One liter polypropylene wide-mouth bottles (Nalge Product No. 2104-0032).

8. Insulated shipping box with carrying strap (17" × 17" × 13"; Cole-Parmer Product No. L-03748-00 and L-03742-30).

9. Miscellaneous — aluminum foil, data card (see **Part 9**), hosecock clamp, surgical gloves, screwdriver or pliers for clamps, waterproof marker.

10. Chemical resistant pump capable of supplying 30 PSI at 3 gal/min and appropriate connectors (for use where garden hose-type pressurized taps for the source or finished water to be monitored are unavailable and for QC samples). Follow the manufacturer's recommendations for pump priming.

MEDIA AND REAGENTS

1. 2% sodium thiosulfate ($Na_2S_2O_3$) — dissolve 100 g of $Na_2S_2O_3$ in a total of 5000 mL dH_2O to prepare a stock solution. Autoclave for 30 min at 121°C.

2. Hydrochloric acid (HCl) — Prepare 0.1, 1 and 5 M solutions by mixing 50, 100 or 50 mL of concentrated HCl with 4950, 900 or 50 mL of dH_2O, respectively. Prepare solutions to be used for adjusting the pH of water samples at least 24 h before use.

PROCEDURE

Operators must wear surgical gloves and avoid conditions that can contaminate a sample with virus. Gloves should be changed after touching human skin or handling components that may be contaminated (e.g., water taps, other environmental surfaces).

Step 1. Purge the water tap to be sampled before connecting the filter apparatus. Continue the purging for 3-3 min or until any debris that has settled in the tap line has cleared. Then turn off the water tap.

Source water sampling must be conducted at the plant intake, before impoundment, chlorination or any other treatment. Finished water sampling must be conducted at the point of entry into the distribution system. If it is necessary to use a pump for sampling, sterilize the pump with chlorine as described in ***Part 5*** *or flush with 20 gal of water to be sampled before each use.*

Step 2. Remove the foil from the backflow regulator (see **Figure VIII-1**) on a regulator module. Loosen the swivel female insert slightly to allow it to turn freely and connect the backflow regulator to the tap. Retighten the swivel female insert. Disconnect the cartridge housing module at the quick connect following the pressure gauge (the insertion point shown in **Figure VIII-1**), if connected, and cover the open ends leading into the modules with sterile foil.

Step 3. Remove the foil from the ends of the discharge module and from the free end of the regulator module. Connect the discharge module to the regulator module. Place the control flow valve or tubing connected to the outlet of the flow control valve into a one liter plastic bottle. Note that the injector module, the prefilter module and the cartridge housing module must not be attached to the apparatus at this stage of the procedure!

Step 4. Slowly turn on the tap and adjust the pressure regulator until the pressure gauge on the regulator module reads 30 PSI. If the tap is incapable of 30 PSI, adjust the regulator to achieve the maximum pressure. Pressures less than 30 PSI will result in a reduced flow rate and thus longer sampling times. Flush the apparatus assembly with at least 20 gal of the water to be sampled. While the system is being flushed, measure the pH, the temperature and the turbidity on the water collecting in and overflowing from the one liter plastic bottle. Record the values onto the **Sample Data Sheet** (see **Part 9**).

The pH meter should be calibrated before each use for the pH range of the water to be sampled.

The turbidity reading may be taken from an in-line turbidimeter connected to the tap being used.

Step 5. If the sample has a pH above 8.0 **or** contains a disinfectant, turn off the water at the tap and disconnect the discharge module from the regulator module. Remove the foil from the

ends of a single injector module (see **Figure VIII-2**) and connect the module to the male quick connect of the regulator module. Reattach the discharge module.

Step 6. If the sample has a pH above 8.0 **and** contains a disinfectant, turn off the water at the tap and disconnect the discharge module from the regulator module. Remove the foil from the ends of a double injector module (see **Figure VIII-2**) and connect the module to the male quick connect of the regulator module. Reattach the discharge module.

Step 7. If an injector module has been added, remove the foil from the injector port(s) and attach the injector tubing to each port. Add a hosecock clamp to each injector tubing and tighten completely to prevent flow into the injector(s). Turn the fine metering adjustment screw on each injector (the smaller screw) clockwise as far as it will go to minimize the flow rate until the injectors are adjusted (note that the injectors were designed to have a minimum flow rate of 20-30 mL/min; thus completely closing the fine metering adjustment screw does not stop the flow). Place the other end of each tubing into the appropriate sterile graduated container containing 0.1 M HCl or 2% thiosulfate. Take care not to touch or contaminate the surfaces of the injector tubing that will be placed in the graduated containers. Slowly turn on the tap again and readjust the pressure regulator, if necessary.

Step 8. If a single injector module has been added, continue to flush the apparatus and adjust the water bypass screw on the injector (the larger adjustment screw) until the pressure gauge on the injector module is about 35% less than the pressure gauge on the regulator module (e.g., 19 PSI when the gauge on the regulator module reads 30 PSI; a minimum of a 35% pressure drop is required to achieve suction). Loosen the hosecock clamp and observe whether suction is occurring. If not, slowly increase the pressure drop until suction starts.

a. If the pH value of the water sample is greater than 8.0, ensure that the injector tubing is placed into a graduated container containing 0.1 M HCl. While continuing to measure the pH in the one liter plastic bottle, adjust the fine metering adjustment screw on the injector to add sufficient HCl to give a pH of 6.5 to 7.5. It may be necessary to use the hosecock clamp to reduce the flow rate to less than 20-30 mL/min or to use a more dilute or concentrated HCl solution with some water samples. When the pH stabilizes at a pH of 6.5 to 7.5, continue with Step 10. Record the adjusted pH onto the **Sample Data Sheet**.

b. If the water to be sampled contains a disinfectant, ensure that the injector tubing is placed into a graduated container containing 2% thiosulfate. Adjust the fine metering adjustment screw on the injector to add thiosulfate at a rate of 10 mL/gal (2.6 mL/L or 30 mL/min at a flow rate of 3 gal/min; note that at this rate, approximately 3-4 L of thio-sulfate solution will be required per sample). When the proper rate is achieved, record the addition of thiosulfate on the **Sample Data Sheet** and continue with Step 10.

Step 9. If a double injector module is being used, continue to flush the apparatus and turn the water bypass screws on each injector clockwise as far as possible. Then turn the water

bypass screws on each regulator one half turn counter clockwise. Continue turning the screws evenly one half turn counter clockwise until the pressure gauge on the double injector module is 35% less than the pressure gauge on the regulator module. Ensure that the tubing from one injector is placed into a graduated container containing 0.1 M HCl and the other into a graduated container containing 2% sodium thiosulfate. Loosen the hosecock clamps. Since there may be slight differences between the injectors and since the pressure reading after the injectors reflects an average pressure drop from both injectors, some additional adjustment of the water bypass screws may be required to obtain suction on each injector. After confirming that each injector is drawing fluid, adjust the flow of HCl and thiosulfate as in Step 8a-8b above. Record the final pH and the addition of thiosulfate on the **Sample Data Sheet** and continue with Step 10.

Step 10. After adjusting the injectors, if required, and flushing the system with at least 20 gal, turn off the flow of water at the sample tap and remove the discharge module. If the water sample has a turbidity greater than 75 NTU, remove the foil from each end of the prefilter module and connect the prefilter module (see **Figure VIII-2**) to the end of the regulator module or to the end of one of the injector modules, if used. Remove the foil from the cartridge housing module and connect it to the end of the regulator module, or to the end of the injector module or the prefilter module, if used. Connect the discharge module to the cartridge housing module.

Step 11. Record the sample number, location, date, time of day and initial gallon (or cubic feet) reading from the water meter onto the **Sample Data Sheet**.

Use the unique utility-specific sample numbers assigned by the ICR Joint Application Design database.

Step 12. Slowly turn on the water with the filter housing placed in an upright position, while pushing the red vent button on top of the filter housing to expel air. When the air is totally expelled from the housing, release the button, and open the sample tap completely. Readjust to 30 PSI, if necessary. Check the thiosulfate usage rate or the pH of the discharged water if an injector(s) is being used and readjust, if necessary.

Step 13. Sample a minimum volume for source water of 200 L (7.1 ft^3, 52.8 gal) and for finished water of 1500 L (53.0 ft^3, 396.3 gal). Samples for source and finished waters must not exceed 300 L (10.6 ft^3, 79.3 gal) and 1800 L (63.6 ft^3, 475.5 gal), respectively. For source water the total amount of sample that can be passed through a filter will depend upon water quality, however, it should be possible to obtain the minimum volume using the procedures described above.

Samples should be monitored periodically during the sampling. If the filter clogs, contact the approved analyst for further instructions. Since the flow rate may change during sampling due to filter clogging, thiosulfate addition and the adjusted pH of the sample must be checked regularly.

Step 14. Turn off the flow of water at the sample tap at the end of the sampling period and record the date, time of day, and final gallon (or cubic feet) reading from the water meter onto the **Sample Data Sheet**. Although the final water meter reading may be affected by the addition of HCl and/or thiosulfate, the effect is considered insignificant and may be ignored.

Step 15. Loosen the swivel female insert on the regulator module and disconnect the backflow regulator from the tap. Disconnect the cartridge housing module and the prefilter housing module, if used from the other modules. Turn the filter housing(s) upside down and allow excess water to flow out as waste water. Turn the housing(s) upright and cover the quick connects on each end of the modules with sterile aluminum foil.

Step 16. Pack the cartridge housing module(s) into an insulated shipping box. Add 6-8 small ice packs (prefrozen at -20°C) around the cartridge housings to keep the sample cool in transit (the number of ice packs may have to be adjusted based upon experience to ensure that the samples remain cold, but not frozen). Drain and add the regulator and injector modules used. Place the **Sample Data Sheet** (protected with a closable plastic bag) in with the sample and ship by overnight courier to the contracted, approved laboratory for virus analysis. Notify the laboratory by phone upon the shipment of sample.

*The approved laboratory will elute virus from the 1MDS filter (and prefilter, if appropriate) and analyze the eluates as described in **Parts 2-3**. After removing the filter, the laboratory will clean, sterilize the apparatus components with chlorine and dechlorinate with sodium thiosulfate as described in **Part 5**. After flushing with sterile dH_2O, a new 1MDS cartridge (and prefilter, if appropriate) will be added, the openings sealed with sterile aluminum foil, and the apparatus returned to the utility for the next sample. The discharge module can be stored at the utility between samplings. Openings should be covered with aluminum foil during storage.*

PART 2 — SAMPLE PROCESSING

QUALITY CONTROL AND PERFORMANCE EVALUATION SAMPLES

*Quality control (QC) and performance evaluation (PE) samples will be shipped to analysts seeking approval (see **Sections III-IV**). PE samples must be successfully analyzed by each analyst participating in the ICR virus monitoring program as part of the initial approval process. After initial approval, each analyst must successfully analyze one QC sample set per sample batch and one PE sample set every month. A QC sample set is comprised of a negative and a positive QC sample. A sample batch consists of all the ICR samples that are analyzed by an analyst during a single week. Each sample batch and its associated QC sample set must be assigned a unique batch number. QC samples do not have to be processed during weekly periods when no ICR samples are processed. QC and PE data should be sent directly to the U.S. EPA as specified in **Section III**.*

QC Samples:

1. Negative QC Sample: Place a sterile 1MDS filter into a standard filter apparatus.
*Process and analyze the 1MDS filter using the **Elution, Organic Flocculation** and **Total Culturable Virus Assay** procedures given below.*

2. Positive QC Sample: Place 40 L of dH_2O into a sterile polypropylene container (Cole-Parmer Product No. G-06063-32) and add 1 mL of a QC stock of attenuated poliovirus containing 200 PFU/mL[2]. Mix and pump the water through a standard filter apparatus containing a 1MDS filter.
*Process and analyze the 1MDS filter using the **Elution, Organic Flocculation** and **Total Culturable Virus Assay** procedures given below.*

PE Samples:
*Process and analyze PE samples according to the **Elution, Organic Flocculation** and **Total Culturable Virus Assay** procedures of this protocol and according to any additional procedures supplied with the samples.*

[2]A QC sample with a titer of 200 PFU/mL will be supplied for the QC tests described in this Section. The titer of this QC sample may be changed before the start or during the testing phase of the ICR. Analysts must use these samples as supplied and not attempt to adjust the titer to 200 PFU/mL. A high titer QC sample will also be shipped to each analyst so that laboratories can develop their own internal QC programs. The high titered sample is not to be used for the QC tests described in this Section.

ELUTION PROCEDURE

The cartridge filters must arrive from the utility in a refrigerated, but not frozen, condition. The arrival condition should be recorded on the ***Sample Data Sheet (Part 9)****. Filters should be refrigerated upon arrival and eluted within 72 h of the start of the sample collection.*

Apparatus and Materials:

1. Positive pressure air or nitrogen source equipped with a pressure gauge.
If the pressure source is a laboratory air line or pump, it must be equipped with an oil filter.

2. Dispensing pressure vessels — 5 or 20 liter capacity (Millipore Corp. Product No. XX67 00P 05 and XX67 00P 20).

3. pH meter with combination-type electrode and an accuracy of at least 0.1 pH unit.

4. Autoclavable inner-braided tubing with screw clamps or quick connects for connecting tubing to equipment.

5. Magnetic stirrer and stir bars.

Media and Reagents:

1. Sodium hydroxide (NaOH) — prepare 1 M and 5 M solutions by dissolving 4 g or 20 g of NaOH in a final volume of 100 mL of dH_2O, respectively.
NaOH solutions may be stored for several months at room temperature.

2. Beef extract V powder (BBL Microbiology Systems Product No. 97531) — prepare buffered 1.5% beef extract by dissolving 30 g of beef extract powder and 7.5 g of glycine (final glycine concentration = 0.05 M) in 1.9 L of dH_2O. Adjust the pH to 9.5 with 1 or 5 M NaOH and bring the final volume to 2 L with dH_2O. Autoclave at 121°C for 15 min and use at room temperature.
Beef extract solutions may be stored for one week at 4°C or for longer periods at -20°C.
Screen each new lot of beef extract before use in the ***Organic Flocculation Concentration Procedure*** *to determine whether virus recoveries are adequate. Perform the screening by spiking one liter of beef extract solution with 1 mL of a diluted QC sample containing 200 PFU/mL. Assay the spiked sample according to the* ***Organic Flocculation*** *and* ***Total Culturable Virus Assay*** *procedures given below. Use a single passage with undiluted sample and sample diluted 1:5 and 1:25 along with an equivalent positive control. The mean recovery of poliovirus for three trials should be at least 50%.*

Procedure:

Place a disinfectant-soaked sponge over vents while releasing trapped air or pressure throughout this procedure to minimize dangers from aerosols.

Step 1. Attach sections of braided tubing (sterilized on inside and outside surfaces with chlorine and dechlorinated with thiosulfate as described in **Part 5**) to the inlet and outlet ports of a cartridge housing module containing a 1MDS filter to be tested for viruses. If a prefilter was used, keep the prefilter and cartridge housing modules connected and attach the tubing to the inlet of the prefilter module and to the outlet of the cartridge housing module.

Step 2. Place the sterile end of the tubing connected to the outlet of the cartridge housing module into a sterile two liter glass or polypropylene beaker.

Step 3. Connect the free end of the tubing from the inlet port of the prefilter or cartridge housing modules to the outlet port of a sterile pressure vessel and connect the inlet port of the pressure vessel to a positive air pressure source. Add pressure to blow out any residual water from the cartridge housing(s). Open the vent/relief valve to release the pressure.

Step 4. Remove the top of the pressure vessel and pour 1000 mL of buffered 1.5% beef extract (pH 9.5, prewarmed to room temperature) into the vessel. Replace the top of the pressure vessel and close its vent/relief valve.

Acceptable alternatives to the use of a pressure vessel include 1) the use of a peristaltic pump and sterile tubing to push the beef extract through the filter and 2) the addition of beef extract directly to the cartridge housing and the use of positive pressure to push the beef extract through the filter.

Step 5. Open the vent/relief valve(s) on the cartridge housing(s) and slowly apply sufficient pressure to purge trapped air from them. Close the vent/relief valve(s) as soon as the buffered beef extract solution begins to flow from it. Turn off the pressure and allow the solution to contact the 1MDS filter for 1 min.

Wipe up spilled liquid with disinfectant-soaked sponge. Carefully observe alternative housings without vents to ensure that all trapped air has been purged.

Step 6. Increase the pressure to force the buffered beef extract solution through the filter(s).

The solution should pass through the 1MDS filter slowly to maximize the elution contact period. When air enters the line from the pressure vessel, elevate and invert the filter housing to permit complete evacuation of the solution from the filters.

Step 7. Turn off the pressure at the source and open the vent/relief valve on the pressure vessel. Place the buffered beef extract from the two liter beaker back into the pressure vessel. Replace the top of the pressure vessel and close its vent/relief valve. Repeat Steps 5 - 6.

Step 8. Turn off the pressure at the source and open the vent/relief valve on the pressure vessel. Thoroughly mix the eluate. Adjust the pH of the eluate to 7.0-7.5 with 1 M HCl. If archiving is not required and if the optional coliphage assay is not performed, measure the volume of the eluate and record it onto the **Virus Data Sheet** as the **Eluate Volume Recovered**. Transfer the **Total Sample Volume** from the **Sample Data Sheet** to the **Adjusted Total Sample Volume** on the **Virus Data Sheet.**

Step 9. If archiving is required or if the optional coliphage assay (see **Section IX. Coliphage Assay**) will be performed, adjust the pH of the eluate to 7.0-7.5 with 1 M HCl. Measure the volume of the adjusted eluate and record it onto the **Virus Data Sheet** as the **Eluate Volume Recovered**. Determine the amount of sample to be used in the coliphage assay by multiplying the **Eluate Volume Recovered** by 0.035. Place a volume equal to the product obtained into a separate container and store at 4°C. If archiving is not required, multiply the **Total Sample Volume** from the **Sample Data Sheet** by 0.965 and record the product as the **Adjusted Total Sample Volume** on the **Virus Data Sheet**.

Step 10. If archiving is required, determine the amount of sample to remove for archiving by multiplying the **Eluate Volume Recovered** by 0.1. Record the product onto the **Virus Data Sheet** as the **Volume of Eluate Archived** and place this volume into a separate container. Freeze[3] the archive sample and ship it to the ICR Laboratory Coordinator, USEPA, TSD, 26 W. Martin Luther King Drive, Cincinnati, OH 45268. Multiply the **Total Sample Volume** from the **Sample Data Sheet** by 0.865 if the optional coliphage assay is performed or by 0.9 if the sample was not assayed for coliphage. Record the product as the **Adjusted Total Sample Volume** on the **Virus Data Sheet**.

Step 11. Proceed to the **Organic Flocculation Concentration Procedure** immediately. If the **Organic Flocculation Concentration Procedure** cannot be undertaken immediately, store the eluate (adjusted to pH 7.0 to 7.5 as described in Step 8b) at 4°C for up to 24 h or for longer periods at -70°C.

ORGANIC FLOCCULATION CONCENTRATION PROCEDURE

Apparatus and Materials:

1. Refrigerated centrifuge capable of attaining 2,500 - 10,000 ×*g* and screw-capped centrifuge bottles with 100 to 1000 mL capacity.

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[3]All freezing of samples and cell cultures throughout this protocol should be performed rapidly by placing vessels in a freezer at -70°C or below or in a dry ice-alcohol bath. Frozen samples and cell cultures should also be thawed rapidly. This may be done by placing vessels in a 37°C waterbath, but vessel caps must not be immersed and vessels should be removed from the waterbath as soon as or just before the last ice crystals melt.

Each bottle must be rated for the relative centrifugal force used.

2. Sterilizing filter — 0.22 µm Acrodisc filter with prefilter (Gelman Sciences Product No. 4525).

Use sterilizing filter stacks on samples that clog commercial filters. Prepare sterilizing filter stacks using 0.22 µm pore size membrane filters (Millipore Corp. Product No. GSWP 47 00) stacked with fiberglass prefilters (Millipore Corp. AP15 47 00 and AP20 47 00).

Stack the prefilters and 0.22 µm membrane into a disc filter holder (Millipore Corp. Product No. SX00 47 00) with the AP20 prefilter on top and 0.22 µm membrane filter on bottom. Disassemble the filter stack after each use to check the integrity of the 0.22 µm filter. Refilter any media filtered with a damaged stack.

Always pass about 10 - 20 mL of sterile beef extract, pH 7.0-7.5 (prepared as above, without pH adjustment), through the filter just before use. This step will reduce virus adsorption onto the filter membranes.

Media and Reagents:

1. Sodium phosphate, dibasic ($Na_2HPO_4 \bullet 7H_2O$) — 0.15 M, pH 9.0 - 9.5 or 7.0 - 7.5.

Dissolve 40.2 g of sodium phosphate in a final volume of 1000 mL dH_2O. The pH of the solution should be between 9.0 - 9.5. Adjust the pH to 9.0 to 9.5 with NaOH, if necessary, or to 7.0 to 7.5 with HCl. Autoclave at 121 ℃ for 15 min.

Procedure:

Minimize foaming (which may inactivate viruses) throughout the procedure by not stirring or mixing faster than necessary to develop a vortex.

Step 1. Place a sterile stir bar into the beaker containing the buffered beef extract eluate from the cartridge filter(s). Place the beaker onto a magnetic stirrer, and stir at a speed sufficient to develop a vortex.

Step 2. Insert a combination-type pH electrode into the beef extract eluate. Add 1 M HCl to the eluate slowly while moving the tip of the pipette in a circular motion away from the vortex to facilitate mixing. Continue adding 1 M HCl until the pH reaches 3.5 ± 0.1 and then stir slowly for 30 min at room temperature.

The pH meter must be standardized at pH 4 and 7. Electrodes must be sterilized before and after each use as described in ***Part 5****.*

A precipitate will form. If pH falls below 3.4, add 1 M NaOH to bring it back to 3.5 ± 0.1. Exposure to a pH below 3.4 may result in some virus inactivation.

Step 3. Remove the electrode from the beaker, and pour the contents of the beaker into a centrifuge bottle. Cap the bottle and centrifuge the precipitated beef extract suspension at 2,500 ×*g* for 15 min at 4°C. Remove and discard the supernatant.

To prevent the transfer of the stir bar into a centrifuge bottle, hold another stir bar or magnet against the bottom of the beaker while decanting the contents. The beef extract suspension will usually have to be divided into several centrifuge bottles.

Step 4. Place a stir bar into the centrifuge bottle that contains the precipitate. Add 30 mL of 0.15 M sodium phosphate, pH 9.0 - 9.5. Place the bottle onto a magnetic stirrer, and stir slowly until the precipitate has dissolved completely.

Since the precipitate may be difficult to dissolve, it can be partially dispersed with a spatula before or during the stirring procedure. It may also be dissolved by repeated pipetting or by shaking at 160 rpm for 20 min on an orbital shaker in place of stirring. When the centrifugation is performed in more than one bottle, dissolve the precipitates in a total of 30 mL and combine into one bottle. If the precipitate is not completely dissolved before proceeding, significant virus loss may occur in Step 5. Because virus loss may also occur by prolonged exposure to pH 9.0-9.5, laboratories that find it difficult to resuspend the precipitate may dissolve it initially in 0.15 M sodium phosphate, pH 7.0 - 7.5. If this variation is used, the pH should be re-adjusted to 9.0-9.5 with 1 M NaOH after the precipitate is completely dissolved and mixed for 10 min at room temperature before proceeding to Step 5.

Step 5. Check the pH and readjust to 9.0-9.5 with 1 M NaOH, as necessary. Remove the stir bar and centrifuge the dissolved precipitate at 4,000 - 10,000 ×*g* for 10 min at 4°C. Remove the supernatant and discard the pellet. Adjust the pH of the supernatant to 7.0-7.5 with 1 M HCl. To remove microbial contamination, load the supernatant into a 50 mL syringe and force it through a sterilizing filter pretreated with beef extract (laboratories may use other approaches to remove contamination, but their effectiveness must be documented). Record the final supernatant (designated the **Final Concentrated Sample Volume**; **FCSV**) on the **Virus Data Sheet** (see **Part 9**).

If the sterilizing filter begins to clog badly, empty the loaded syringe into the bottle containing the unfiltered supernatant, fill the syringe with air, and inject air into filter to force any residual sample from it. Continue the filtration procedure with another filter.

Step 6. Determine the volume of sample that must be assayed. This volume is at least 100 L for source water or 1000 L for finished water and is designated the **Volume of Original Water Sample Assayed**[4] (**D**). Record the value of **D** on the **Virus Data Sheet**. Calculate the **Assay Sample Volume** (**S**) for source and finished water samples using the formula:

$$S = \frac{D}{ATSV} \times FCSV$$

[4]Analytical laboratories assaying more than the required volume must use the actual volume to be assayed in the calculation. See **Part 8** for examples of the calculations used in this protocol.

where **ATSV** is the **Adjusted Total Sample Volume** from the **Virus Data Sheet**. The **Assay Sample Volume** is the volume of the **Final Concentrated Sample** that represents 100 L of source water or 1000 L of finished water. Record the **Assay Sample Volume** onto the **Virus Data Sheet**. Prepare a subsample (subsample 1) containing a volume 0.55 times the **Assay Sample Volume**. Prepare a second subsample (subsample 2) containing a volume that is 0.67 times the **Assay Sample Volume**. Divide the **Final Concentrated Sample** from QC and PE samples into two equal subsamples. Calculate the **Assay Sample Volume** for these samples by multiplying **FCSV** by 0.4. Label each subsample with appropriate sampling information for identification. Hold any portion of the sample that can be assayed within 24 h at 4°C and freeze all other portions at -70°C.

Final Concentrated Samples, subsamples, PE and QC samples processed to this point by a laboratory not doing the virus assay must be frozen at -70°C immediately and then shipped on dry ice to the laboratory approved for the virus assay.

PART 3 — TOTAL CULTURABLE VIRUS ASSAY

QUANTAL ASSAY

Apparatus and Materials:

1. Incubator capable of maintaining the temperature of cell cultures at 36.5 ± 1°C.

2. Sterilizing filter — 0.22 µm (Costar Product No. 140666).
Always pass about 10 - 20 mL of 1.5% beef extract, pH 7.0-7.5, through the filter just before use to minimize virus adsorption to the filter.

Media and Reagents:

1. Prepare BGM cell culture test vessels using standard procedures.
*BGM cells are a continuous cell line derived from African Green monkey kidney cells and are highly susceptible to many enteric viruses (Dahling et al., 1984; Dahling and Wright, 1986). The characteristics of this line were described by Barron et al. (1970). The use of BGM cells for recovering viruses from environmental samples was described by Dahling et al. (1974). For laboratories with no experience with virus recovery from environmental samples, the media and procedures described by Dahling and Wright (1986) and given in **Part 4** are recommended for maximum sensitivity.*
*EPA will supply an initial culture of BGM cells at about passage 117 to all laboratories seeking approval. Upon receipt, laboratories must prepare an adequate supply of frozen BGM cells using standard procedures to replace working cultures that become contaminated or lose virus sensitivity. A **Procedure for Preservation of the BGM Cell Line** is given in **Part 4**. Only BGM cells from the U.S. EPA and between passage 117 and 250 may be used for virus monitoring under the ICR.*

Sample Inoculation and CPE Development:
Cell cultures used for virus assay are generally found to be at their most sensitive level between the third and sixth days after their most recent passage. Those older than seven days should not be used.

Step 1. Identify cell culture test vessels by coding them with an indelible marker. Return the cell culture test vessels to a 36.5 ± 1°C incubator and hold at that temperature until the cell monolayer is to be inoculated.

Step 2. Decant and discard the medium from cell culture test vessels. Wash the test vessels with a balanced salt solution or maintenance medium without serum using a wash volume of at least 0.06 mL/cm^2 of surface area. Rock the wash medium over the surface of each monolayer several times and then decant and discard the wash medium.
Do not disturb the cell monolayer.

Step 3. Determine the **Inoculum Volume** by dividing the **Assay Sample Volume** by 20. Record the **Inoculum Volume** onto the **Virus Data Sheet**. The **Inoculum Volume** should be no greater than 0.04 mL/cm^2 of surface area. If the **Inoculum Volume** is greater than 0.04 mL/cm^2, use larger culture vessels.

Step 4. Inoculate each BGM cell culture test vessel with an amount of assay control or water sample equal to the **Inoculum Volume** and record the date of inoculation on the **Sample Data Sheet** (see **Part 9**).

Avoid touching either the cannula or the pipetting device to the inside rim of the cell culture test vessels to avert the possibility of transporting contaminants to the remaining culture vessels.

*For ease of inoculation, a sufficient quantity of 0.15 M Na_2HPO_4, pH 7.0 - 7.5, may be added to the **Inoculum Volume** to give a more usable working **Inoculation Volume** (e.g., 1.0 mL). For example, if an **Inoculum Volume** of 0.73 mL is to be placed onto 10 vessels, then 10.5 × (1 - 0.73 mL) = 2.84 mL of sodium phosphate, pH 7.0-7.5 could be added to 10.5 × 0.73 = 7.67 mL of subsample. Each milliliter of the resulting mixture will contain the required **Inoculum Volume**.*

a. Total Culturable Virus Assay Controls:

Run a negative and positive assay control with every group of subsamples inoculated onto cell cultures.

i. **Negative Assay Control**: Inoculate a BGM culture with a volume of sodium phosphate, pH 7.0 - 7.5, equal to the **Inoculation Volume**. This culture will serve as negative control for the tissue culture quantal assay. If any **Negative Assay Control** develops cytopathic effects (CPE), all subsequent assays of water samples should be halted until the cause of the positive result is determined.

ii. **Positive Assay Control**: Dilute attenuated poliovirus type 3 (from the high titered QC stock) in sodium phosphate, pH 7.0 - 7.5, to give a concentration of 20 PFU per **Inoculation Volume**. Inoculate a BGM culture with an amount of diluted virus equal to the **Inoculation Volume**. This control will provide a measure for continued sensitivity of the cell cultures to virus infection. Additional positive control samples may be prepared by adding virus to a small portion of the final concentrated sample and/or by using additional virus types. If any **Positive Assay Control** fails to develop CPE, all subsequent assays of water samples should be halted until the cause of the negative result is determined. It may be necessary to thaw and use an earlier passage of the BGM cell line supplied by the U.S. EPA.

b. Inoculation of Water Samples

i. Rapidly thaw subsample 1, if frozen, and inoculate an amount equal to the **Inoculum Volume** onto each of 10 cell cultures. If there is no evidence for cytotoxicity and if at least three cell cultures are negative for CPE after seven days (see below), thaw subsample 2 and inoculate an amount equal to the **Inoculum Volume** onto each of 10 additional cultures.

Hold a thawed subsample for no more than 4 h at 4 ℃. Warm the subsample to room temperature just before inoculation.

A small portion of the Final Concentrated Sample may by inoculated onto cultures several days before inoculating subsample 1 as a control for cytotoxicity.

ii. If cytotoxicity is not a problem and more than seven cultures are positive for CPE after seven days, prepare five- and twenty five-fold dilutions of subsample 2. To prepare a 1:5 dilution, add a volume equal to 0.1334 times the **Assay Sample Volume** (amount "a") to a volume of 0.15 M sodium phosphate (pH 7.0-7.5) equal to 0.5334 times the **Assay Sample Volume** (amount "b"). After mixing thoroughly, prepare a 1:25 dilution by adding amount "a" of the 1:5 diluted sample to amount "b" of 0.15 M sodium phosphate (pH 7.0-7.5). Using an amount equal to the **Inoculum Volume**, inoculate 10 cell cultures each with undiluted subsample 2, subsample 2 diluted 1:5 and subsample 2 diluted 1:25, respectively. Freeze the remaining portions of the 1:25 dilution at -70°C until the sample results are known. If the inoculated cultures are all positive, thaw the remaining 1:25 dilution and prepare 1:125, 1:625 and 1:3125 dilutions by transferring amount "a" of each lower dilution to amount "b" of sodium phosphate as described above. Inoculate 10 cultures each with the additional dilutions and freeze the remaining portion of the 1:3125 dilution. Continue the process of assaying higher dilutions until at least one test vessel at the highest dilution tested is negative. Higher dilutions can also be assayed along with the initial undiluted to 1:25 dilutions if it is suspected that the water to be tested contains more than 500 most probable number (MPN) of infectious total culturable virus units per 100 L.

iii. If subsample 1 is cytotoxic, then five cell cultures should be inoculated with Final Concentrated Sample using the same volume required for subsample 1 and the procedures described in the **Reduction of Cytotoxicity in Sample Concentrates** section below. If these procedures remove cytotoxicity, inoculate subsample 2 using the procedures for removal of cytotoxicity and 10 cultures each with undiluted sample, sample diluted 1:5 and sample diluted 1:25 as in Step 4bii above. If the procedures fail to remove cytotoxicity, write for advice on how to proceed to the ICR Laboratory Coordinator, U.S. EPA, Office of Ground Water and Drinking Water, Technical Support Division, 26 W. Martin Luther King Drive, Cincinnati, OH 45268.

*A maximum of 60 and 580 MPN units per 100 L can be demonstrated by inoculating a total of 20 cultures with the undiluted **Assay Sample Volume** from source water or a total of 10 cultures each with undiluted sample and sample diluted 1:5 and 1:25, respectively.*

c. Inoculation of QC and PE Samples: prepare five-fold dilutions of subsample 1 for each negative QC sample as described in Step 4bii. Prepare five- and twenty five-fold dilutions for each positive QC and PE sample. Inoculate 10 cultures with undiluted subsample and each diluted subsample using an amount of inoculum equal to the **Inoculum Volume**.

Use subsample 2 only as a backup for problems with the analysis of subsample 1.

Step 5. Rock the inoculated cell culture test vessels gently to achieve uniform distribution of inoculum over the surface of the cell monolayers. Place the cell culture test vessels on a level stationary surface at room temperature so that the inoculum remains distributed evenly over the cell monolayer.

Step 6. Continue incubating the inoculated cell cultures for 80 - 120 min to permit viruses to adsorb onto and infect cells.

It may be necessary to rock the vessels every 15-20 min or to keep them on a mechanical rocking platform during the adsorption period to prevent cell death in the middle of the vessels from dehydration.

Step 7. Add liquid maintenance medium (see Item 2 of **Vessels and Media for Cell Growth** in **Part 4** for recommended medium) and incubate at 36.5 ± 1°C.

Warm the maintenance medium to 36.5 ± 1 °C before placing it onto cell monolayers. Add the medium to the side of the cell culture vessel opposite the cell monolayer. Avoid touching any pipetting devices used to the inside rim of the culture vessels to avert the possibility of transporting contaminants to the remaining vessels. The cultures may be re-fed with fresh maintenance medium after 4 - 7 days.

Step 8. Examine each culture microscopically for the appearance of CPE daily for the first three days and then every couple of days for a total of 14 days.

CPE may be identified as cell disintegration or as changes in cell morphology. Rounding-up of infected cells is a typical effect seen with enterovirus infections. However, uninfected cells round-up during mitosis and a sample should not be considered positive unless there are significant clusters of rounded-up cells over and beyond what is observed in the uninfected controls. Photomicrographs demonstrating CPE appear in the reference by Malherbe and Strickland-Cholmley (1980).

Step 9. Freeze cultures at -70°C when more than 75% of the monolayer shows signs of CPE. Freeze all remaining negative cultures, including controls, after 14 days.

Step 10. Thaw all the cultures to confirm the results of the previous passage. Filter at least 10% of the medium from each vessel that was positive for CPE or that appeared to be bacterially contaminated through separate 0.22 µm sterilizing filters. Then inoculate another BGM culture with 10% of the medium from the previous passage for each vessel, including those that were negative. Repeat Steps 7 - 8.

Confirmation passages may be performed in small vessels or multiwell trays, however, it may be necessary to distribute the inoculum into several vessels or wells to insure that the ***Inoculum Volume*** *is less than or equal to 0.04 mL/cm^2 of surface area.*

Step 11. Score cultures that developed CPE in both the first and second passages as confirmed positives. Cultures that show CPE in only the second passage must be passaged a third time along with the negative controls according to Steps 9 - 10. Score cultures that develop CPE in both the second and third passages as confirmed positives.

Cultures with confirmed CPE may be stored in a -70 ℃ freezer for research purposes or for optional identification tests.[5]

Virus Quantitation:

Step 1. Record the total number of confirmed positive and negative cultures for each subsample onto the **Total Culturable Virus Data Sheet** (**Part 9**). Do not include the results of tests for cytotoxicity!

Step 2. Transfer the number of cultures inoculated and the confirmed number of positive cultures from the **Total Culturable Virus Data Sheet** for each subsample to the **Quantitation of Total Culturable Virus Data Sheet**. If dilutions are not required, add the values to obtain a total undiluted count for each sample. Calculate the MPN/mL value (M_m) and the upper (CL_{um}) and lower (CL_{lm}) 95% confidence limits using the total undiluted count. If dilutions are required, calculate the MPN/mL value and 95% confidence limits using only the subsample 2 values. Place the values obtained onto the **Quantitation of Total Culturable Virus Data Sheet**. The MPNV computer program supplied by the U.S. EPA must be used for the calculation of all MPN values and confidence limits.

Step 3. Calculate the MPN per 100 liter value (M_l) of the original water sample according the formula:

$$M_l = \frac{100\ M_m S}{D}$$

[5]For more information see Chapter 12 (May 1988 revision) of Berg *et al*. (1984).

where S equals the **Assay Sample Volume** and D equals the **Volume of Original Water Sample Assayed** (the values for S and D can be found on the **Virus Data Sheet**). Record the value of M_l onto the **Virus Data Sheet**.[6]

Step 4. Calculate the lower 95% confidence limit per 100 liter value (CL_l) for each water sample according to the formula:

$$CL_l = \frac{100\ CL_{lm}S}{D}$$

where CL_{lm} is the lower 95% confidence limit per milliliter from the **Quantitation of Total Culturable Virus Data Sheet**. Calculate the upper 95% Confidence Limit per 100 liter value (CL_u) according to the formula:

$$CL_u = \frac{100\ CL_{um}S}{D}$$

where CL_{um} is the upper 95% confidence limit per milliliter from the **Quantitation of Total Culturable Virus Data Sheet**. Record the limit per 100 liter values on the **Virus Data Sheet**.

Step 5. Calculate the total MPN value and the total 95% confidence limit values for each QC and PE sample by multiplying the values per milliliter by S and dividing by 0.4.

REDUCTION OF CYTOTOXICITY IN SAMPLE CONCENTRATES

The procedure described in this section may result in a significant titer reduction and should be applied only to inocula known to be or expected to be toxic.

Media and Reagents:

1. Washing solution.

Dissolve 8.5 g of NaCl in a final volume of 980 mL of dH_20. Autoclave the solution at 121 ℃ for 15 min. Cool to room temperature. Add 20 mL serum to the sterile salt solution. Mix thoroughly. Store the washing solution at 4 ℃ for up to three months or at -20 ℃.

The volume of the NaCl washing solution required will depend on the number of bottles to be processed and the cell surface area of the vessels used for the quantal assay.

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[6]Use significant figures when reporting all results throughout the protocol (see APHA, 1995, p. 1-17).

Procedure for Cytotoxicity Reduction:

Step 1. Decant and save the inoculum from inoculated cell culture vessels after the adsorption period (Step 5 of **Sample Inoculation and CPE Development**). Add 0.25 mL of the washing solution for each cm^2 of cell surface area into each vessel.

Warm the washing solution to 36.5 ± 1 °C before placing on cell monolayer. Add the washing solution to the side of the cell culture vessel opposite the cell monolayer. Avoid touching any pipetting devices used to the inside rim of the culture vessels to avert the possibility of transporting contaminants to the remaining vessels.

The inocula saved after the adsorption period should be stored at -70 °C for subsequent treatment and may be discarded when cytotoxicity is successfully reduced.

Step 2. Gently rock the washing solution gently across the cell monolayer a minimum of two times. Decant and discard the spent washing solution without disturbing the cell monolayer.

It may be necessary to rock the washing solution across the monolayer more than twice if sample is oily and difficult to remove from the cell monolayer surface.

Step 3. Continue with Step 7 of the procedure for **Sample Inoculation and CPE Development**.

If this procedure fails to reduce cytotoxicity with a particular type of water sample, backup samples may be diluted 1:2 to 1:4 before repeating the procedure. This dilution requires that two to four times more culture vessels be used. Dilution alone may sufficiently reduce cytotoxicity of some samples without washing. Alternatively, the changing of liquid maintenance medium at the first signs of cytotoxicity may prevent further development.

*Determine cytotoxicity from the initial daily macroscopic examination of the appearance of the cell culture monolayer by comparing the negative control from Step 4ai and the positive control from Step 4aii of the procedure for **Sample Inoculation and CPE Development** with the test samples from Step 4b). Cytotoxicity should be suspected when the cells in the test sample develop CPE before its development on the positive control.*

PART 4 — CELL CULTURE PREPARATION AND MAINTENANCE

PREPARATION OF CELL CULTURE MEDIUM

General Principles:

1. Equipment care — Carefully wash and sterilize equipment used for preparing media before each use.

2. Disinfection of work area — Thoroughly disinfect surfaces on which the medium preparation equipment is to be placed.

3. Aseptic technique — Use aseptic technique when preparing and handling media or medium components.

4. Dispensing filter-sterilized media — To avoid post-filtration contamination, dispense filter-sterilized media into storage containers through clear glass filling bells in a microbiological laminar flow hood. If a hood is unavailable, use an area restricted solely to cell culture manipulations.

5. Coding media — Assign a lot number to and keep a record of each batch of medium or medium components prepared. Place the lot number, the date of preparation, the expiration date, and the initials of the person preparing the medium on each bottle.

6. Sterilization of $NaHCO_3$-containing solutions — Sterilize media and other solutions that contain $NaHCO_3$ by positive pressure filtration.
Negative pressure filtration of such solutions increases the pH and reduces the buffering capacity.

7. Antibiotic solutions prepared in-house must be filter sterilized with 0.22 µm membrane filters. It is important that the recommended antibiotic levels not be exceeded during the planting of cells, as cultures are particularly sensitive to excessive concentrations at this stage. Antibiotic stock solutions should be placed in screw-capped containers and stored at -20°C until needed. Once thawed, they may be refrozen; however, repeated freezing and thawing of these stock solutions should be avoided by freezing them in quantities that are sufficient to support a week's cell culture work.

Apparatus and Materials:

1. Glassware, Pyrex (Corning Product No. 1395).
Storage vessels must be equipped with airtight closures.

2. Disc filter holders — 142 mm or 293 mm diameter (Millipore Product No. YY30 142 36 and YY30 293 16).

Use only positive pressure type filter holders.

3. Sterilizing filter stacks — 0.22 µm pore size (Millipore Product No. GSWP 142 50 and GSWP 293 25). Fiberglass prefilters (Millipore AP15 142 50 or AP15 293 25, and AP20 142 50 or AP20 293 25).

Stack AP20 and AP15 prefilters and 0.22 µm membrane filter into a disc filter holder with AP20 prefilter on top and 0.22 µm membrane filter on bottom.

Always disassemble the filter stack after use to check the integrity of the 0.22 µm filter. Refilter any media filtered with a damaged stack.

4. Positively-charged cartridge filter — 10 inch (Zeta plus TSM, Cuno Product No. 45134-01-600P). Cartridge housing with adaptor for 10 inch cartridge (Millipore Product No. YY16 012 00).

5. Culture capsule filter (Gelman Sciences Product No. 12170).

6. Cell culture vessels — Pyrex, soda or flint glass or plastic bottles and flasks or roller bottles (e.g., Brockway Product No. 1076-09A, 1925-02, Corning Product No. 25100-25, 25110-75, 25120-150, 25150-1750).

Vessels must be made from clear glass or plastic to allow observation of the cultures and be equipped with airtight closures. Plastic vessels must be treated by the manufacturer to allow cells to adhere properly.

7. Screw caps, black with rubber liners (Brockway Product No. 24-414).

Caps for larger culture bottles usually supplied with bottles.

8. Roller apparatus (Bellco Glass Product No. 7730).

Required only if roller bottles are used for maintenance of stock cultures.

9. Waterbath set at 56 ± 1°C.

10. Light microscope, with conventional light source, equipped with lenses to provide 40X, 100X, and 400X total magnification.

11. Inverted light microscope equipped with lenses to provide 40X, 100X, and 400X total magnification.

12. Phase contrast counting chamber (hemocytometer) (Curtin Matheson Scientific Product No. 158-501).

13. Conical centrifuge tubes — 50 and 250 mL capacity.

14. Rack for tissue culture tubes (Bellco Product No. 2028).

15. Bottles, aspirator-type with tubing outlet — 2,000 mL capacity.
Bottles for use with pipetting machine.

16. Storage vials — 2 mL capacity.
Vials must withstand temperatures to -70 ℃.

Media and Reagents:

1. Sterile fetal calf, gamma globulin-free newborn calf or iron-supplemented calf serum, certified free of viruses, bacteriophage and mycoplasma.
Test each lot of serum for cell growth and toxicity before purchasing. Serum should be stored at -20 ℃ for long-term storage. Upon thawing, each bottle must be heat-inactivated in a waterbath set at 56 ± 1 ℃ for 30 min and stored at 4 ℃ for short term use.

2. Trypsin, 1:250 powder (Difco Laboratories Product No. 0152-15-9) or trypsin, 1:300 powder (Becton Dickinson Microbiology Systems Product No. 12098).

3. EDTA (Fisher Scientific Product No. S657-500).

4. Fungizone (amphotericin B, Sigma Product No. A-9528), penicillin G (Sigma Product No. P-3032), streptomycin sulfate (ICN Biomedicals Product No. 100556), tetracycline hydrochloride (ICN Biomedicals Product No. 103011).
Use antibiotics of at least tissue culture grade.

5. Eagle's minimum essential medium (MEM) with Hanks' salts and L-glutamine, without sodium bicarbonate (Life Technologies Product No. 410-1200).

6. Leibovitz's L-15 medium with L-glutamine (Life Technologies Product No. 430-1300).

7. Trypan blue (Sigma Chemical Product No. T-6146).

8. Dimethyl sulfoxide (DMSO; Sigma Chemical Product No. D-2650).

Media Preparation Recipes:
The conditions specified by the supplier for storage and expiration dates of commercially available media should be strictly observed.

1. Procedure for the preparation of 10 L of EDTA-trypsin.
The procedure described is used to dislodge cells attached to the surface of culture bottles and flasks. This reagent, when stored at 4 ℃, retains its working strength for at least four

months. The amount of reagent prepared should be based on projected usage over a four month period.

Step a. Add 30 g of trypsin (1:250) or 25 g of trypsin (1:300) to 2 L of dH_2O in a six liter flask containing a three inch stir bar. Place the flask onto a magnetic stirrer and mix the trypsin solution rapidly for a minimum of 1 h.

The trypsin remains cloudy.

Step b. Add 4 L of dH_2O and a three-inch stir bar into a 20 liter clear plastic carboy. Place the carboy onto a magnetic stirrer and stir at a speed sufficient to develop a vortex while adding the following chemicals: 80 g NaCl, 12.5 g EDTA, 50 g glucose, 11.5 g $Na_2HPO_4 \bullet 7H_2O$, 2.0 g KCl, and 2.0 g KH_2PO_4.

Each chemical does not have to be completely dissolved before adding the next one.

Step c. Add an additional 4 L dH_2O to the carboy and continue mixing until all the chemicals are completely dissolved.

Step d. Add the 2 L of trypsin from Step 2a to the solution from Step 2c and mix for a minimum of 1 h. Adjust the pH of the EDTA-trypsin reagent to 7.5 - 7.7.

Step e. Filter the reagent under pressure through a filter stack and store the filtered reagent in tightly stoppered or capped containers at 4°C.

*The cartridge prefilter (**Item 4** of **Apparatus and Materials**) can be used in line with the culture capsule sterilizing filter (**Item 5**) as an alternative to a filter stack (**Item 3**).*

2. Procedure for the preparation of 10 L of MEM/L-15 medium.

Step a. Place a three inch stir bar and 4 L of dH_2O into a 20 liter clear plastic carboy.

Step b. Place the carboy onto a magnetic stirrer. Stir at a speed sufficient to develop a vortex and then add the contents of a five liter packet of L-15 medium to the carboy. Rinse the medium packet with three washes of 200 mL each of dH_2O and add the rinses to the carboy.

Step c. Mix until the medium is evenly dispersed.

L-15 medium may appear cloudy as it need not be totally dissolved before proceeding to Step d.

Step d. Add 3 L of dH_2O to the carboy and the contents of a five liter packet of MEM medium to the carboy. Rinse the MEM medium packet with three washes of 200 mL each of dH_2O and add the rinses to the carboy. Add 800 mL of dH_2O and 7.5 g of $NaHCO_3$ and continue mixing for an additional 60 min.

Step e. Transfer the MEM/L-15 medium to a pressure can and filter under positive pressure through a 0.22 µm sterilizing filter. Collect the medium in volumes appropriate for the culturing of BGM cells (e.g., 900 mL in a one liter bottle) and store in tightly stoppered or capped containers at 4°C for up to two months.

Note that the volume of the MEM/L-15 medium adds up to only 9 L to allow for the addition of serum to a final concentration of 10%.

3. Procedure for preparation of 100 mL of trypan blue solution.

The procedure is used in the direct determination of the viable cell counts of the BGM stock cultures. As trypan blue is on the U.S. EPA suspect carcinogen list, particular care should be taken in its preparation and use so as to avoid skin contact or inhalation. The wearing of rubber gloves during preparation and use is recommended.

Step a. Add 0.5 g of trypan blue to 100 mL of dH_2O in a 250 mL flask. Swirl the flask until the trypan blue is completely dissolved.

Step b. Sterilize the solution by autoclaving at 121°C for 15 min and store in a screw-capped container at room temperature.

4. Preparation of 100 mL of penicillin-streptomycin stock solution containing 100,000 units/mL of penicillin and 100,000 µg/mL of streptomycin.

Step a. Add 10,000,000 units of penicillin G and 10 g of streptomycin sulfate to a 250 mL flask containing 100 mL of dH_2O. Mix the contents of the flasks on magnetic stirrer until the antibiotics are dissolved.

Step b. Sterilize the antibiotics by filtration through a 0.22 µm membrane filter and dispense in 10 mL volumes into screw-capped containers.

5. Preparation of 50 mL of tetracycline stock solution.

Step a. Add 1.25 g of tetracycline hydrochloride powder and 3.75 g of ascorbic acid to a 125 mL flask containing 50 mL of dH_2O. Mix the contents of the flask on a magnetic stirrer until the antibiotic is dissolved.

Step b. Sterilize the antibiotic by filtration through a 0.22 µm membrane filter and dispense in 5 mL volumes into screw-capped containers.

6. Preparation of 25 mL of amphotericin B (fungizone) stock solution.

Step a. Add 0.125 g of amphotericin B to a 50 mL flask containing 25 mL of dH_2O. Mix the contents of the flask on a magnetic stirrer until the antibiotic is dissolved.

Step b. Sterilize the antibiotic by filtration through a 0.22 µm membrane filter and dispense in 2.5 mL volumes into screw-capped containers.

PREPARATION AND PASSAGE OF BGM CELL CULTURES

A microbiological biosafety cabinet should be used to process cell cultures. If a hood is not available, cell cultures should be prepared in controlled facilities used for no other purposes. Viruses or other microorganisms must not be transported, handled, or stored in rooms used for cell culture transfer.

Vessels and Media for Cell Growth:

1. The BGM cell line grows readily on the inside surfaces of glass or specially treated, tissue culture grade plastic vessels. Flat-sided, glass bottles (16 to 32 oz or equivalent growth area), 75 or 150 cm^2 plastic cell culture flasks, and 690 cm^2 glass or 850 cm^2 plastic roller bottles are usually used for the maintenance of stock cultures. Flat-sided bottles and flasks that contain cells in a stationary position are incubated with the flat side (cell monolayer side) down. If available, roller bottles and roller apparatus units are preferable to flat-sided bottles and flasks because roller cultures require less medium than flat-sided bottles per unit of cell monolayer surface area. Roller apparatus rotation speed should be adjusted to one-half revolution per minute to ensure that cells are constantly bathed in growth medium.

2. Growth and maintenance media should be prepared on the day they will be needed. Prepare growth medium by supplementing MEM/L-15 medium with 10% serum and antibiotics (100 mL of serum, 1 mL of penicillin-streptomycin stock, 0.5 mL of tetracycline stock and 0.2 mL of fungizone stock per 900 mL of MEM/L-15). Prepare maintenance medium by supplementing MEM/L-15 with antibiotics and 2% or 5% serum (20 or 50 mL of serum, antibiotics as above for growth medium and 80 or 50 mL of dH_2O, respectively). Use maintenance media with 2% serum for CPE development.

General Procedure for Cell Passage:

Pass stock BGM cell cultures at approximately seven day intervals using growth medium.

Step 1. Pour spent medium from cell culture vessels, and discard the medium.

A gauze-covered beaker may be used to collect spent medium to prevent splatter. Autoclave all media that have been in contact with cells or that contain serum before discarding.

Step 2. Add a volume of warm EDTA- trypsin reagent equal to 40% of the volume of medium that was discarded in Step 1.

*See **Table VIII-1** for the amount of reagents required for commonly used vessel types. Warm the EDTA-trypsin reagent to 36.5 ± 1 ℃ before placing it onto cell monolayers.*

Step 3. Allow the EDTA-trypsin reagent to remain in contact with cells at room temperature until the cell monolayer can be shaken loose from the inner surface of the cell culture vessel.

To prevent cell damage, the EDTA-trypsin reagent should remain in contact with the cells no longer than 5 min.

Table VIII-1. Guide for Preparation of BGM Stock Cultures

Vessel Type	Volume of EDTA-Trypsin (mL)	Volume of Medium (mL)[a]	Total No. Cells to Plate per Vessel
16 oz glass flat bottles	10	25	2.5×10^6
32 oz glass flat bottles	20	50	5.0×10^6
75 cm^2 plastic flat flask	12	30	3.0×10^6
150 cm^2 plastic flat flask	24	60	6.0×10^6
690 cm^2 glass roller bottle	40	100	7.0×10^6
850 cm^2 plastic roller bottle	48	120	8.0×10^7

[a]Serum requirements: growth medium contains 10% serum; maintenance medium contains 2-5% serum. Antibiotic requirements: penicillin-streptomycin stock solution, 1.0 mL/liter; tetracycline stock solution, 0.5 mL/liter; fungizone stock solution, 0.2 mL/liter.

Step 4. Pour the suspended cells into centrifuge tubes or bottles.

To facilitate collection and resuspension of cell pellets, use tubes or bottles with conical bottoms. Centrifuge tubes and bottles used for this purpose must be able to withstand the g-force applied.

Step 5. Centrifuge cell suspension at 1,000 ×*g* for 10 min to pellet cells. Pour off and discard the supernatant.

Do not exceed this speed as cells may be damaged or destroyed.

Step 6. Suspend the pelleted cells in growth medium (see Item 2 of **Vessels and Media for Cell Growth**) and perform a viable count on the cell suspension according to the **Procedure for Performing Viable Cell Counts** section below.

Resuspend pelleted cells in a sufficient volume of medium to allow thorough mixing of the cells (to reduce sampling error) and to minimize the significance of the loss of the 0.5 mL of cell suspension required for the cell counting procedure. The quantity of medium used for resuspending pelleted cells varies from 50 to several hundred milliliters, depending upon the volume of the individual laboratory's need for cell cultures.

Step 7. Dilute the cell suspension to the appropriate final cell concentration with growth medium and dispense into cell culture vessels with a pipet, a Cornwall syringe or a Brewer- type pipetting machine dispenser.

Table VIII-2. Preparation of Virus Assay Cell Cultures

Vessel Type	Volume of Medium* (mL)	Final Cell Count per Vessel
1 oz glass bottle	4	9.0×10^5
25 cm^2 plastic flask	10	3.5×10^6
6 oz glass bottle	15	5.6×10^6
75 cm^2 plastic flask	30	1.0×10^7
16 mm × 150 mm tubes	2	4.0×10^4

*Serum requirements: growth medium contains 10% serum. Antibiotic requirements: penicillin- streptomycin stock solution, 1.0 mL/liter; tetracycline stock solution, 0.5 mL/liter; fungizone stock solution, 0.2 mL/liter.

Calculate the dilution factor requirement using the cell count and the cell and volume parameters given in ***Table VIII-1*** *for stock cultures and in* ***Table VIII-2*** *for virus assay cultures.*

As a general rule, the BGM cell line should be split at a 1:2 ratio for passages 117 to 150 and a 1:3 ratio for passages 151 to 250. To plant two hundred 25 cm^2 cell culture flasks weekly from cells between 151 and 250 passages would require the preparation of six roller bottles (surface area of 690 cm^2 each): The contents of two to prepare the next batch of six roller bottles and the contents of the other four to prepare the 25 cm^2 flasks.

Step 8. Except during handling operations, maintain BGM cells at 36.5 ± 1°C in airtight cell culture vessels.

Step 9. Replace growth medium with maintenance medium containing 2% serum when cell monolayers become 95 to 100% confluent (usually three to four days after seeding with an appropriate number of cells). Replace growth medium that becomes acidic before the monolayers become 95 to 100% confluent with maintenance medium containing 5% serum. The volume of maintenance medium should equal the volume of the discarded growth medium.

Procedure For Performing Viable Cell Counts:

Step 1. Add 0.5 mL of cell suspension (or diluted cell suspension) to 0.5 mL of 0.5% trypan blue solution in a test tube.

To obtain an accurate cell count, the optimal total number of cells per hemocytometer section should be between 20 and 50. This range is equivalent to between 6.0×10^5 and 1.5×10^6 cells per mL of cell suspension. Thus, a dilution of 1:10 (0.5 mL of cells in 4.5 mL of growth medium) is usually required for an accurate count of a cell suspension.

Step 2. Disperse cells by repeated pipetting.

Avoid introducing air bubbles into the suspension, because air bubbles may interfere with subsequent filling of the hemocytometer chambers.

Step 3. With a capillary pipette, carefully fill a hemocytometer chamber on one side of a slip-covered hemocytometer slide. Rest the slide on a flat surface for about 1 min to allow the trypan blue to penetrate the cell membranes of nonviable cells.

Do not under or over fill the chambers.

Step 4. Under 100X total magnification, count the cells in the four large corner sections and the center section of the hemocytometer chamber.

Include in the count cells lying on the lines marking the top and left margins of the sections, and ignore cells on the lines marking the bottom and right margins. Trypan blue is excluded by living cells. Therefore, to quantify viable cells, count only cells that are clear in color. Do not count cells that are blue.

Step 5. Calculate the average number of viable cells in each mL of cell suspension by totaling the number of viable cells counted in the five sections, multiplying this sum by 2000, and where necessary, multiplying the resulting product by the reciprocal of the dilution.

PROCEDURE FOR PRESERVATION OF BGM CELL LINE

An adequate supply of frozen BGM cells must be available to replace working cultures that are used only periodically or become contaminated or lose virus sensitivity. Cells have been held at -70 ℃ for more than 15 years with a minimum loss in cell viability.

Preparation of Cells for Storage:

The procedure described is for the preparation of 100 cell culture vials. Cell concentration must be at least 2×10^6 per mL.

The actual number of vials to be prepared should be based upon line usage and the anticipated time interval requirement between cell culture start-up and full culture production.

Step 1. Prepare cell storage medium by adding 10 mL of DMSO to 90 mL of growth medium (see **Item 2** of **Vessels and Media for Cell Growth**). Sterilize the resulting cell storage medium by passage through a 0.22 µm sterilizing filter.

Collect sterilized medium in a 250 mL flask containing a stir bar.

Step 2. Harvest BGM cells from cell culture vessels as directed in Steps 1 to 5 of **General Procedures for Cell Passage**. Count the viable cells as described above and resuspend them in the cell storage medium at a concentration of at least 2×10^6 cells per mL.

Step 3. Place the flask containing suspended cells on a magnetic stirrer and slowly mix for 30 min. Dispense 1 mL volumes of cell suspension into 2 mL capacity vials.

Procedure for Freezing Cells:

The freezing procedure requires slow cooling of the cells with the optimum rate of -1 ℃ per min. A slow cooling rate can be achieved using the following method or by using the recently available freezing containers (e.g., Nalge Product No. 5100-0001) as recommended by the manufacturers.

Step 1. Place the vials in a rack and place the rack in refrigerator at 4°C for 30 min, then in a -20°C freezer for 30 min, and finally in a -70°C freezer overnight. The transfers should be made as rapidly as possible.

To allow for more uniform cooling, wells adjoining each vial should remain empty.

Step 2. Rapidly transfer vials into boxes or other containers for long-term storage.

To prevent substantial loss of cells during storage, temperature of cells should be kept constant after -70°C has been achieved.

Procedure for Thawing Cells:

Cells must be thawed rapidly to decrease loss in cell viability.

Step 1. Place vials containing frozen cells into a 36.5 ± 1°C water bath and agitate vigorously by hand until all ice has melted. Sterilize the outside surface of the vials with 0.5% I_2 in 70% ethanol.

Step 2. Add BGM cells to either 6 oz tissue culture bottles or 25 cm^2 tissue culture flasks containing an appropriate volume of growth medium (see **Table VIII-2**). Use two vials of cells for 6 oz bottles and one vial for 25 cm^2 flasks.

Step 3. Incubate BGM cells at 36.5 ± 1°C. After 18 to 24 h replace the growth medium with fresh growth medium and then continue the incubation for an additional five days. Pass and maintain the new cultures as directed above.

PART 5 — STERILIZATION AND DISINFECTION

GENERAL GUIDELINES

1. Use aseptic techniques for handling test waters, eluates and cell cultures.

2. Sterilize apparatus and containers that will come into contact with test waters and all solutions that will be added to test waters unless otherwise indicated. Thoroughly clean all items before final sterilization using laboratory standard operating procedures.

3. Sterilize all contaminated materials before discarding.

4. Disinfect all spills and splatters.

STERILIZATION TECHNIQUES

Solutions:

1. Sterilize all solutions, except those used for cleansing, standard buffers, hydrochloric acid (HCl), sodium hydroxide (NaOH), and disinfectants by autoclaving them at 121°C for at least 15 min.

The HCl and NaOH solutions and disinfectants used are self-sterilizing. When autoclaving buffered beef extract, use a vessel large enough to accommodate foaming.

Autoclavable Glassware, Plasticware, and Equipment:

*Water speeds the transfer of heat in larger vessels during autoclaving and thereby speeds the sterilization process. Add dH_2O to vessels in quantities indicated in **Table VIII-3**. Lay large vessels on their sides in the autoclave, if possible, to facilitate the displacement of air in the vessels by flowing steam.*

1. Cover the openings into autoclavable glassware, plasticware, and equipment loosely with aluminum foil before autoclaving. Autoclave at 121°C for at least 30 min.

Glassware may also be sterilized in a dry heat oven at a temperature of 170 °C for at least 1 h.

2. Sterilize stainless steel vessels (dispensing pressure vessel) in an autoclave at 121°C for at least 30 min.

Vent-relief valves on vessels so equipped must be open during autoclaving and closed immediately when vessels are removed from autoclave.

3. Presterilize 1MDS filter cartridges and prefilter cartridges by wrapping the filters in Kraft paper and autoclaving at 121°C for 30 min.

4. Sterilize instruments, such as scissors and forceps, by immersing them in 95% ethanol and flaming them between uses.

Table VIII-3. Water Quantity to be Added to Vessels Before Autoclaving

Vessel Size (liter)	Quantity of dH_2O (mL)
2 and 3	25
4	50
8	100
24	500
54	1000

Chlorine Sterilization:

Sterilize pumps, plasticware (filter housings) and tubing that cannot withstand autoclaving, and vessels that are too large for the autoclave by chlorination. Prefilters, but not 1MDS filters, may be presterilized with chlorine as an alternative to autoclaving. Filter apparatus modules should be disinfected by sterilization and then cleaned according to laboratory standard operating procedures before final sterilization.

1. Media and Reagents

   a. 0.1% chlorine (HOCl) — add 19 mL of household bleach (Clorox, The Clorox Co.) to 900 mL of dH_2O and adjust the pH of the solution to 6-7 with 1 M HCl. Bring to 1 liter with dH_2O.

2. Procedures

Ensure that the solutions come in full contact with all surfaces when performing these procedures.

   a. Sterilize filter apparatus modules, injector tubing and plastic bags for transporting injector tubing by recirculating or immersing the items in 0.1% chlorine for 30 min. Drain the chlorine solution from objects being sterilized. Dechlorinate using a solution containing 2.5 mL of 2% sterile sodium thiosulfate per liter of sterile dH_2O.

   b. Thoroughly rinse pH electrodes after each use to remove particulates. Sterilize before and after each use by immersing the tip of the electrode in 0.1% chlorine for at least 1 min. Dechlorinate the electrode as in Step 2a above. Rinse with sterile dH_2O.

PROCEDURE FOR VERIFYING STERILITY OF LIQUIDS

Do not add antibiotics to media or medium components until after their sterility has been demonstrated. The BGM cell line used should be checked every six months for mycoplasma contamination according to test kit instructions. Cells that are contaminated should be discarded.

Media and Reagents:

1. Mycoplasma testing kit (Irvine Scientific Product No. T500-000). Use as directed by the manufacturer.

2. Thioglycollate medium (Difco Laboratories Product No. 0257-01-9). Prepare broth medium as directed by the manufacturer.

Verifying Sterility of Small Volumes of Liquids:

Step 1. Inoculate 1 mL portions of the material to be tested for sterility into tubes containing 9 mL of thioglycollate broth by stabbing the inoculum into the broth. Incubate at 36.5 ± 1°C.

Step 2. Examine the inoculated broth daily for seven days to determine whether growth of contaminating organisms has occurred.

Containers holding the thioglycollate medium must be tightly sealed before and after the medium is inoculated.

Visual Evaluation of Media for Microbial Contaminants:

Step 1. Incubate either the entire stock of prepared media or portions taken during preparation that represent at least 5% of the final volume at 36.5 ± 1°C for at least one week before use.

Step 2. Visually examine and discard any media that lose clarity.

A clouded condition that develops in the media indicates the occurrence of contaminating organisms.

CONTAMINATED MATERIALS

1. Autoclave contaminated materials for at least 30 min at 121°C. Be sure that steam can enter contaminated materials freely.

2. Many commercial disinfectants do not adequately kill enteric viruses. To ensure thorough disinfection, disinfect spills and other contamination on surfaces with either a solution of 0.5% iodine in 70% ethanol (5 g I_2 per liter) or 0.1% chlorine. The iodine solution has the advantage of drying more rapidly on surfaces than chlorine, but may stain some surfaces.

PART 6 — BIBLIOGRAPHY AND SUGGESTED READING

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PART 7 — VENDORS

The vendors listed below represent one possible source for required products. Other vendors may supply the same or equivalent products.

American Type Culture Collection
12301 Parklawn Dr.
Rockville, MD 20852
(800) 638-6597

Baxter Diagnostics, Scientific Products Div.
1430 Waukegan Rd.
McGaw Park, IL 60085
(800) 234-5227

BBL Microbiology Systems: products may be ordered through several major scientific supply houses

Becton Dickinson Microbiology Systems
250 Schilling Circle
Cockeysville, MD 21030
(410) 771-0100 (Ask for a local distributor)

Bellco Glass
340 Edrudo Rd.
Vineland, NJ 08360
(800) 257-7043

Brockway: products may be ordered through Continental Glass & Plastics

Cincinnati Valve and Fitting Co.
3710 Southern Ave.
Cincinnati, OH 45227
(513) 272-1212

Cole-Parmer Instrument Co.
7425 N. Oak Park Ave.
Niles, IL 60714
(800) 323-4340

Continental Glass & Plastics
841 W. Cermak Rd.
Chicago, IL 60608
(312) 666-2050

Corning: products may be ordered through most major scientific supply houses

Costar Corp.
7035 Commerce Circle
Pleasanton, CA 94588
(800) 882-7711

Cuno, Inc.
400 Research Parkway
Meriden, CT 06450
(800)243-6894

Curtin Matheson Scientific
P.O. Box 1546
Houston, TX 77251
(713) 820-9898

DEMA Engineering Co.
10014 Big Bend Blvd.
Kirkwood, MO 63122
(800) 325-3362

Difco Laboratories
P.O. Box 331058
Detroit, MI 48232
(800) 521-0851 (Ask for a local distributor)

Fisher Scientific
711 Forbes Ave.
Pittsburgh, PA 15219
(800) 766-7000

Gelman Sciences
600 S. Wagner Rd.
Ann Arbor, MI 48103
(800) 521-1520

ICN Biomedicals
3300 Hyland Ave.
Costa Mesa, CA 92626
(800) 854-0530

Irvine Scientific
2511 Daimler Street
Santa Ana, CA 92705
(800) 437-5706

Life Technologies
P.O. Box 68
Grand Island, NY 14072
(800) 828-6686

Millipore Corp.
397 Williams St.
Marlboro, MA 01752
(800) 225-1380

Nalge Co.
P.O. Box 20365
Rochester, NY 14602
(716) 586-8800 (Ask for a local distributor)

Neptune Equipment Co.
520 W. Sharon Rd.
Forest Park, OH 45240
(800) 624-6975

OMEGA Engineering, Inc.
P.O. Box 4047
Stamford, CT 06907
(800) 826-6342

Plast-o-matic Valves, Inc.
1384 Pompton Ave
Cedar Grove, NJ 07009
(201) 256-3000 (Ask for a local distributor)

Parker Hannifin Corp.
Commercial Filters Div.
1515 W. South St., Lebanon, IN 46052
(317) 482-3900

Ryan Herco
2509 N. Naomi St.
Burbank, CA 91504
(800) 848-1141

Sigma Chemical
P.O. Box 14508
St. Louis, MO 63178
(800) 325-3010

United States Plastic Corp.
1390 Neubrecht Rd.
Lima, OH 45801
(800) 537-9724

Watts Regulator
Box 628
Lawrence, MA 01845
(508) 688-1811

PART 8 — EXAMPLES

EXAMPLE 1

A source water sample of 211.98 L was collected at the Sampleville Water Works on 5/1/95 and shipped by overnight courier to CEPOR Laboratories. CEPOR Laboratories processed the sample on 5/2/95. After elution, the pH of the beef extract V eluate was adjusted to 7.3 with 1 M HCl. The volume of the pH-adjusted eluate, 980 mL, was recorded. Volumes of 34.3 mL (980 × 0.035) and 98.0 mL (980 × 0.1) were removed for the **Coliphage Assay** (**Section IX**) and for archiving, respectively. An **Adjusted Total Sample Volume** (ATSV) was then calculated by multiplying 211.98 L × 0.865. An ATSV of 183 L was recorded on the **Virus Data Sheet**.

The sample was immediately processed by the **Organic Flocculation Concentration Procedure**. Following centrifugation at 4,000 ×*g*, the supernatant was adjusted to pH 7.3 and passed through a sterilizing filter. A **Final Concentrated Sample Volume** (FCSV) of 28.0 mL was obtained.

The **Assay Sample Volume** was calculated using the formula:

$$ASSAY\ SAMPLE\ VOLUME\ (S) = \frac{D}{ATSV} \times FCSV$$

where D is the **Volume of Original Water Sample Assayed** (i.e., 100 L for source water or 1000 L for finished water). Thus the **Assay Sample Volume** for Sampleville-01 is:

$$S = \frac{100\ liters}{183\ liters} \times 28.0\ ml = 15.3\ ml$$

The 15.3 mL is the volume of the **Final Concentrated Sample** that must be inoculated onto tissue culture and that represents 100 L of the source water.

Two subsamples were prepared from the **Final Concentrated Sample**. **Subsample 1** was prepared by placing 0.55 × 15.3 mL = 8.4 mL into a separate container. **Subsample 2** was prepared by placing 0.67 × 15.3 mL = 10.2 mL into a third container. Although only 0.5 × 15.3 = 7.65 mL (representing 50 L of source water) must be inoculated onto tissue culture flasks for each subsample, the factor “0.55" was used for **subsample 1** to account for unrecoverable losses associated with removing a subsample from its container. The factor “0.67" was used for **subsample 2** to account for losses associated with the container and to provide additional sample for the preparation of dilutions, if required.

Subsample 2 and the remaining portions of the **Final Concentrated Sample** were frozen at -70°C.

The **inoculation volume** was calculated to be 15.3 mL ÷ 20 = 0.76 mL per flask. To make the inoculation procedure more convenient, it was decided to dilute **subsample 1** so that 1.0 mL of inoculum contained an amount of **subsample 1** equal to the **inoculum volume**. To do this, 10.5 × (1.00 - 0.76) = 2.52 mL of 0.15 M $Na_2HPO_4 \bullet 7H_2O$, pH 7.3, was added to 10.5 × 0.76 = 7.98 mL of **subsample 1**. One milliliter of diluted **subsample 1** was then inoculated onto each of ten 25 cm^2 flasks of BGM cells at passage 123. A **negative control** was prepared by inoculating a flask with 1.0 mL of 0.15 M $Na_2HPO_4 \bullet 7H_2O$, pH 7.3. A **positive control** was prepared by inoculating a flask with 1.0 mL of 0.15 M $Na_2HPO_4 \bullet 7H_2O$, pH 7.3 containing 200 PFU/mL of attenuated poliovirus type 3. Following adsorption, 9.0 mL of maintenance medium was added and the cultures were incubated at 36.5°C. These cultures and those described below were observed for CPE as described in the protocol and positive cultures were frozen when 75% of a flask showed signs of CPE.

On May 9th five flasks inoculated with **subsample 1** and the positive control showed signs of CPE. Because fewer than eight flasks inoculated with **subsample 1** showed CPE, 10 additional 25 cm^2 flasks of BGM cells at passage 124 were inoculated with 1.0 mL each of **subsample 2** diluted in the same manner as **subsample 1**. Another **negative control** and **positive control** were also prepared and inoculated.

By May 16th a total of seven flasks inoculated with **subsample 1** showed signs of CPE. The flasks that had not been previously frozen were now frozen at -70°C and then all flasks were thawed. Several milliliters of fluid from each of the eight positive flasks (seven samples plus the positive control) were passed through a sterilizing filter. Twelve flasks of BGM cells at passage 125 were inoculated with one milliliter of the supernatant from either negative cultures or from filtered positive cultures.

By May 23rd a total of five flasks from **subsample 2** showed signs of CPE. All flasks were frozen, thawed and then passaged as described for **subsample 1** using BGM cells at passage 126.

By May 30th only six flasks from the second passage of **subsample 1** and the positive control showed CPE. Thus one culture from the 1st passage failed to confirm in the second pass and a value of 6 was recorded in the **Number of Replicates with CPE** column of the **Total Culturable Virus Data Sheet**. The flasks were then discarded.

On June 6th seven flasks (the five original plus two new flasks) from the second passage of **subsample 2** demonstrated CPE. The two new flasks and controls were frozen at -70°C, thawed and passaged a third time as described above using BGM cells at passage 127. All other flasks were discarded.

By June 12 the positive control and the two third passage flasks had developed CPE. All flasks were discarded at this time (the flasks would have been examined until 6/20 if at least one had remained negative). A value of 7 was recorded into the **Number of Replicates with CPE** column of the **Total Culturable Virus Data Sheet**.

The MPN software program supplied by the U.S. EPA was used to calculate the MPN/mL and 95% confidence limit values. "I. SIZE OF INOCULUM VOLUME (mL)" on the main screen was changed from 1 to 0.76. "A. PROCEED WITH DATA INPUT" was pressed followed by "ENTER" to overwrite the existing output file. Alternatively, "NO" could have been entered and the output file renamed. The number of positive replicates, "13," was then entered. Following the calculation by the program, the MPN and 95% Confidence Limit values were recorded onto the **Quantitation of Total Culturable Virus Data Sheet**. The program was exited by pressing "I. EXIT THE PROGRAM."

The MPN per 100 liter value (M_l) was calculated according to the formula:

$$M_l = \frac{100\ M_m S}{D} = \frac{100 \times 1.38 \times 15.3}{100} = 21.1$$

where M_m is the MPN value per milliliter from the **Quantitation of Total Culturable Virus Data Sheet**, S is the **Assay Sample Volume** and D is the **Volume of Original Water Sample Assayed** (S and D are obtained from the **Virus Data Sheet**).

The Lower 95% Confidence Limit per 100 liter (CL_l) was calculated according to the formula:

$$CL_l = \frac{100\ CL_{lm} S}{D} = \frac{100 \times 0.70 \times 15.3}{100} = 10.7$$

where CL_{lm} is the lower 95% confidence limit per milliliter from the **Quantitation of Total Culturable Virus Data Sheet**.

The Upper 95% Confidence Limit per 100 liter (CL_u) was calculated according to the formula:

$$CL_u = \frac{100\ CL_{um} S}{D} = \frac{100 \times 2.27 \times 15.3}{100} = 34.7$$

where CL_{um} is the upper 95% confidence limit per milliliter from the **Quantitation of Total Culturable Virus Data Sheet**.

SAMPLE DATA SHEET

SAMPLE NUMBER: Sampleville-01

UTILITY NAME: Sampleville Water Works

UTILITY ADDRESS: 1 Water Street
CITY: Sampleville **STATE:** OH **ZIP:** 45999

SAMPLER'S NAME: Mr. Brian Hall

WATER TEMPERATURE: 23.5 °C **TURBIDITY:** 3.6 NTU

WATER pH: 7.8

ADJUSTED WATER pH: NA

THIOSULFATE ADDED: (CHECK) _ YES X NO

INIT. METER READING: 6048.10 **CHECK UNITS:** X gallons _ ft^3
date: 5/1/95 **time:** 9 am

FINAL METER READING: 6104.10 **CHECK UNITS:** X gallons _ ft^3
date: 5/1/95 **time:** 9:30 am

TOTAL SAMPLE VOLUME: 211.98 L

(Final-Initial meter readings × 3.7854 (for readings in gallons) or × 28.316 (for readings in ft^3))

SHIPMENT DATE: 5/1/95

CONDITION ON ARRIVAL: Cold/Not frozen

COMMENTS:

VIRUS DATA SHEET

SAMPLE NUMBER: SAMPLEVILLE-01

ANALYTICAL LABORATORY NAME: CEPOR LABORATORIES

ANALYTICAL LABORATORY ADDRESS: 42 RUECKERT ST.
CITY: CINCINNATI STATE: OH ZIP: 45219

ADJUSTED TOTAL SAMPLE VOLUME (ATSV):[1]	**183**	**L**
DATE ELUTED: 5/2/95	**TIME: 10 am**	
ELUATE VOLUME RECOVERED:	**980**	**mL**
VOLUME OF ELUATE ARCHIVED:	**98.0**	**mL**
DATE CONCENTRATED: 5/2/95	**TIME: 1 pm**	
FINAL CONCENTRATED SAMPLE VOLUME (FCSV):	**28.0**	**mL**
ASSAY SAMPLE VOLUME (S):	**15.3**	**mL**
VOLUME OF ORIGINAL WATER SAMPLE ASSAYED (D):	**100**	**L[2]**
INOCULUM VOLUME:	**0.76**	**mL**

DATES ASSAYED BY CPE:	**1st Passage**	**2nd Passage**	**3rd Passage (If necessary)**
Subsample 1:	**5/2/95**	**5/16/95**	
Subsample 2:	**5/9/95**	**5/23/95**	**6/6/95**

	95% CONFIDENCE LIMITS
MPN/100 L[3]: 21	**LOWER: 11 UPPER: 35**

COMMENTS:

ANALYST: B.G. Moore

[1]Enter the Total Sample Volume times 0.965 if a coliphage sample is taken, times 0.9 if archiving is required, times 0.865 if a coliphage sample is taken and archiving is required or times 1 if a coliphage sample is not taken and archiving is not required.
[2]Must be at least 100 L for source water and 1000 L for finished water.
[3]Value calculated from the Quantitation of Total Culturable Virus form as described in the Virus Quantitation section of Part 3.

TOTAL CULTURABLE VIRUS DATA SHEET

SAMPLE #: Sampleville-01

	Total Number of Replicates					
	Subsample 1			Subsample 2		
Sample	Inoculated	Without CPE	With CPE	Inoculated	Without CPE	With CPE
1st Passage Neg. Cont.	1	1	0	1	1	0
Pos. Cont.	1	0	1	1	0	1
Undiluted	10	3	7	10	5	5
1:5 Dil.						
1:25 Dil.						
2nd Passage[1] Neg. Cont.	1	1	0	1	1	0
Pos. Cont.	1	0	1	1	0	1
Undiluted	10	4	6	10	3	7
1:5 Dil.						
1:25 Dil.						
3rd Passage[2] Neg. Cont.				1	1	0
Pos. Cont.				1	0	1
Undiluted				2	0	2
1:5 Dil.						
1:25 Dil.						

[1]A portion of medium from each 1st passage vessel, including controls, must be repassaged for conformation. The terms "Undiluted," "1:5 Dilution" and "1:25 Dilution" under the 2nd and 3rd Passage headings refer to the original sample dilutions for the 1st passage. If higher dilutions are used, record the data from the three highest dilutions showing positive results and place the actual dilution amount in the sample column.

[2]Samples that were negative on the first passage and positive on the 2nd passage must be passaged a third time for conformation. If a third passage is required, all controls must be passaged again.

QUANTITATION OF TOTAL CULTURABLE VIRUS DATA SHEET

SAMPLE NUMBER: Sampleville-01

Sample	Number Replicates inoculated	Number with CPE	MPN/mL[1]	95% Confidence Limits	
				Lower	Upper
Undiluted Samples			1.38	0.70	2.27
Subsample 1	10	6			
Subsample 2	10	7			
Total Undiluted	20	13			
Subsample 2 results (Dilutions Required)					
Undiluted					
1:5 Dilution					
1:25 Dilution					

[1]Use the values recorded in the Total Undiluted row to calculate the MPN/mL result and confidence limits when dilutions are not required. If dilutions are required, base the calculation upon the values recorded in the Undiluted, 1:5 Diluted and 1:25 Diluted rows for subsample 2. If higher dilutions are used for subsample 2, record the data from the three highest dilutions showing positive results and place the actual dilution amount in the sample column. The MPN/mL and 95% Confidence Limit values must be obtained using the computer program supplied by the U.S. EPA.

EXAMPLE 2

A source water sample of 200.63 L was collected at the Sampleville Water Works on 6/5/95 and shipped by overnight courier to CEPOR Laboratories. CEPOR Laboratories processed the sample on 6/6/95. After elution, the pH was adjusted to 7.3. A volume of 985 mL of pH-adjusted eluate was obtained and 34.5 mL (985 mL × 0.035) was removed for the **Coliphage Assay** (**Section IX**). Archiving was not required. An **Adjusted Total Sample Volume** of 194 L (200.63 L × 0.965) was recorded on the **Virus Data Sheet**.

The sample was immediately processed by the **Organic Flocculation Concentration Procedure**. Following centrifugation at 4,000 ×*g*, the supernatant was adjusted to pH 7.3 and passed through a sterilizing filter. A **Final Concentrated Sample Volume** of 32.0 mL was obtained, giving an **Assay Sample Volume** for Sampleville-02 of:

$$S = \frac{100\ liters}{194\ liters} \times 32.0\ ml = 16.5\ ml$$

Subsample 1 was prepared by placing 0.55 × 16.5 mL = 9.1 mL into a separate container. **Subsample 2** was prepared by placing 0.67 × 16.5 mL = 11.1 mL into a third container. **Subsample 2** and the remaining portions of the **Final Concentrated Sample** were frozen at -70°C.

Subsample 1 was inoculated onto each of ten 25 cm^2 flasks of BGM cells at passage 127 using an **inoculation volume** of 16.5 mL ÷ 20 = 0.82 mL per flask. A **negative control** was prepared by inoculating a flask with 0.82 mL of 0.15 M $Na_2HPO_4 \bullet 7H_2O$, pH 7.3. A **positive control** was prepared by inoculating a flask with 0.82 mL of 0.15 M $Na_2HPO_4 \bullet 7H_2O$, pH 7.3 containing 241.0 PFU/mL (200.0 PFU/0.82 mL) of attenuated poliovirus type 3. Following adsorption, 9.18 mL of maintenance medium was added and the cultures were incubated at 36.5°C.

On June 13 nine flasks inoculated with **subsample 1** and the positive control showed signs of CPE. After thawing **subsample 2**, a 1:5 dilution was prepared by mixing 0.1334 × 16.5 = 2.20 mL of **subsample 2** with 0.5334 × 16.5 = 8.80 mL of 0.15 M $Na_2HPO_4 \bullet 7H_2O$, pH 7.3. A 1:25 dilution was prepared by mixing 2.20 mL of the 1:5 dilutions with 8.80 mL of 0.15 M $Na_2HPO_4 \bullet 7H_2O$, pH 7.3. Ten 25 cm^2 flasks of BGM cells at passage 128 were then inoculated with 0.82 mL each of undiluted **subsample 2**. Ten flasks were inoculated with 0.82 mL each of **subsample 2** diluted 1:5 and ten flasks were inoculated with 0.82 mL each of **subsample 2** diluted 1:25. Another **negative control** and **positive control** were also prepared and inoculated.

By June 20 all 10 flasks inoculated with **subsample 1** showed signs of CPE and were repassaged as described in example 1.

By June 27 all 10 flasks inoculated with undiluted **subsample 2** had developed CPE. Eight flasks inoculated with the 1:5 dilution of **subsample 2** and four flasks inoculated with the 1:25 dilution of **subsample 2** demonstrated CPE. All flasks were re-passaged as described for example 1.

By July 5th all 10 flasks from the second passage of **subsample 1** were confirmed as positive and were discarded.

By July 11th all 10 flasks inoculated with the second passage of undiluted **subsample 2** had developed CPE. The eight positive flasks from the 1st passage of the 1:5 dilution of **subsample 2** were positive in the second passage. Three flasks inoculated with the second passage of the 1:25 dilution of **subsample 2** remained positive.

The MPN software program supplied by the U.S. EPA was used to calculate the MPN/mL and 95% confidence limit values. After the main screen appeared, "G. NUMBER OF DILUTIONS" was changed from 1 to 3. "H. NUMBER OF REPLICATES PER DILUTION" was changed from 20 to 10 and "I. SIZE OF INOCULUM VOLUME (mL)" was changed from 1 to 0.82. "A. PROCEED WITH DATA INPUT" was pressed followed by "ENTER" to overwrite the existing output file. The number of positive replicates per dilution, "10, 8, and 3" was entered with the values separated by spaces. Following program calculations, the MPN/mL and 95% Confidence Limit values/mL were recorded onto the **Quantitation of Total Culturable Virus Data Sheet**. The program was exited by pressing "I. EXIT THE PROGRAM."

The MPN per 100 liter value (M_l) was calculated according to the formula:

$$M_l = \frac{100\ M_m S}{D} = \frac{100 \times 10.15 \times 16.5}{100} = 167$$

where M_m is the MPN value per milliliter from the **Quantitation of Total Culturable Virus Data Sheet**, S is the **Assay Sample Volume** and D is the **Volume of Original Water Sample Assayed** (S and D are obtained from the **Virus Data Sheet**).

The Lower 95% Confidence Limit per 100 liter (CL_l) was calculated according to the formula:

$$CL_l = \frac{100\ CL_{lm} S}{D} = \frac{100 \times 5.04 \times 16.5}{100} = 83.1$$

where CL_{lm} is the lower 95% confidence limit per milliliter from the **Quantitation of Total Culturable Virus Data Sheet**.

The Upper 95% Confidence Limit per 100 liter (CL_u) was calculated according to the formula:

$$CL_u = \frac{100\ CL_{um}S}{D} = \frac{100 \times 18.25 \times 16.5}{100} = 301$$

where CL_{um} is the upper 95% confidence limit per milliliter from the **Quantitation of Total Culturable Virus Data Sheet**.

SAMPLE DATA SHEET

SAMPLE NUMBER: Sampleville-02

UTILITY NAME: Sampleville Water Works

UTILITY ADDRESS: 1 Water Street
CITY: Sampleville **STATE:** OH **ZIP:** 45999

SAMPLER'S NAME: Mr. Brian Hall

WATER TEMPERATURE: 26.5 °C **TURBIDITY:** 2.3 NTU

WATER pH: 7.7

ADJUSTED WATER pH: NA

THIOSULFATE ADDED: (CHECK) _ YES X NO

INIT. METER READING: 6129.3 **CHECK UNITS:** X gallons _ ft^3
date: 6/5/95 **time:** 8:30 am

FINAL METER READING: 6182.3 **CHECK UNITS:** X gallons _ ft^3
date: 6/5/95 **time:** 9:00 am

TOTAL SAMPLE VOLUME: 200.63 L
(Final-Initial meter readings × 3.7854 (for readings in gallons)
or × 28.316 (for readings in ft^3))

SHIPMENT DATE: 6/5/95

CONDITION ON ARRIVAL: Cold/Not frozen

COMMENTS:

VIRUS DATA SHEET

SAMPLE NUMBER: SAMPLEVILLE-02

ANALYTICAL LABORATORY NAME: CEPOR LABORATORIES

ANALYTICAL LABORATORY ADDRESS: 42 RUECKERT ST.
CITY: CINCINNATI STATE: OH ZIP: 45219

ADJUSTED TOTAL SAMPLE VOLUME (ATSV): [1] 194 L

DATE ELUTED: 6/6/95 | **TIME: 9:50 am**

ELUATE VOLUME RECOVERED: 985 mL

VOLUME OF ELUATE ARCHIVED: 0 mL

DATE CONCENTRATED: 6/6/95 | **TIME: 1 pm**

FINAL CONCENTRATED SAMPLE VOLUME (FCSV): 32.0 mL

ASSAY SAMPLE VOLUME (S): 16.5 mL

VOLUME OF ORIGINAL WATER SAMPLE ASSAYED (D): 100 L[2]

INOCULUM VOLUME: 0.82 mL

DATES ASSAYED BY CPE:	1st Passage	2nd Passage	3rd Passage (If necessary)
Subsample 1:	6/6/95	6/20/95	
Subsample 2:	6/13/95	6/27/95	

MPN/100 L[3]: 167

95% CONFIDENCE LIMITS
LOWER: 83 UPPER: 301

COMMENTS:

ANALYST: B.G. Moore

[1]Enter the Total Sample Volume times 0.965 if a coliphage sample is taken, times 0.9 if archiving is required, times 0.865 if a coliphage sample is taken and archiving is required or times 1 if a coliphage sample is not taken and archiving is not required.
[2]Must be at least 100 L for source water and 1000 L for finished water.
[3]Value calculated from the Quantitation of Total Culturable Virus form as described in the Virus Quantitation section of Part 3.

TOTAL CULTURABLE VIRUS DATA SHEET

SAMPLE #: Sampleville-02

	Total Number of Replicates					
	Subsample 1			Subsample 2		
Sample	Inoculated	Without CPE	With CPE	Inoculated	Without CPE	With CPE
1st Passage Neg. Cont.	1	1	0	1	1	0
Pos. Cont.	1	0	1	1	0	1
Undiluted	10	0	10	10	0	10
1:5 Dil.				10	2	8
1:25 Dil.				10	6	4
2nd Passage[1] Neg. Cont.	1	1	0	1	1	0
Pos. Cont.	1	0	1	1	0	1
Undiluted	10	0	10	10	0	10
1:5 Dil.				10	2	8
1:25 Dil.				10	7	3
3rd Passage[2] Neg. Cont.						
Pos. Cont.						
Undiluted						
1:5 Dil.						
1:25 Dil.						

[1]A portion of medium from each 1st passage vessel, including controls, must be repassaged for conformation. The terms "Undiluted," "1:5 Dilution" and "1:25 Dilution" under the 2nd and 3rd Passage headings refer to the original sample dilutions for the 1st passage. If higher dilutions are used, record the data from the three highest dilutions showing positive results and place the actual dilution amount in the sample column.

[2]Samples that were negative on the first passage and positive on the 2nd passage must be passaged a third time for conformation. If a third passage is required, all controls must be passaged again.

QUANTITATION OF TOTAL CULTURABLE VIRUS DATA SHEET

SAMPLE NUMBER: Sampleville-02

Sample	Number Replicates inoculated	Number with CPE	MPN/mL[1]	95% Confidence Limits	
				Lower	Upper
Undiluted Samples			10.15	5.04	18.25
Subsample 1	10	10			
Subsample 2					
Total Undiluted	NA	NA			
Subsample 2 results (Dilutions Required)					
Undiluted	10	10			
1:5 Dilution	10	8			
1:25 Dilution	10	3			

[1]Use the values recorded in the Total Undiluted row to calculate the MPN/mL result and confidence limits when dilutions are not required. If dilutions are required, base the calculation upon the values recorded in the Undiluted, 1:5 Diluted and 1:25 Diluted rows for subsample 2. If higher dilutions are used for subsample 2, record the data from the three highest dilutions showing positive results and place the actual dilution amount in the sample column. The MPN/mL and 95% Confidence Limit values must be obtained using the computer program supplied by the U.S. EPA.

PART 9 — DATA SHEETS [7]

[7]Copies of all Data Sheets are available upon request in WordPerfect for Windows, version 6.1 format. Send requests to the ICR Laboratory Coordinator, USEPA, TSD, 26 W. Martin Luther King Drive, Cincinnati, OH 45268.

SAMPLE DATA SHEET			
SAMPLE NUMBER:			
UTILITY NAME:			
UTILITY ADDRESS: **CITY:**		**STATE:**	**ZIP:**
SAMPLER'S NAME:			
WATER TEMPERATURE:	**°C**	**TURBIDITY:**	**NTU**
WATER pH:			
ADJUSTED WATER pH:			
THIOSULFATE ADDED:	**(CHECK) __ YES __ NO**		
INIT. METER READING: **date:**		**CHECK UNITS:** **time:**	**__gallons __ft^3**
FINAL METER READING: **date:**		**CHECK UNITS:** **time:**	**__gallons __ft^3**
TOTAL SAMPLE VOLUME:		**L**	
(Final-Initial meter readings × 3.7854 (for readings in gallons) or × 28.316 (for readings in ft^3))			
SHIPMENT DATE:			
CONDITION ON ARRIVAL:			
COMMENTS:			

VIRUS DATA SHEET			
SAMPLE NUMBER:			
ANALYTICAL LABORATORY NAME:			
ANALYTICAL LABORATORY ADDRESS: **CITY:** **STATE:** **ZIP:**			
ADJUSTED TOTAL SAMPLE VOLUME (ATSV): [1] **L**			
DATE ELUTED:		**TIME:**	
ELUATE VOLUME RECOVERED: **mL**			
VOLUME OF ELUATE ARCHIVED: **mL**			
DATE CONCENTRATED:		**TIME:**	
FINAL CONCENTRATED SAMPLE VOLUME (FCSV): **mL**			
ASSAY SAMPLE VOLUME (S): **mL**			
VOLUME OF ORIGINAL WATER SAMPLE ASSAYED (D): **L**			
INOCULUM VOLUME: **mL**			
DATES ASSAYED BY CPE:	**1st Passage**	**2nd Passage**	**3rd Passage (If necessary)**
Subsample 1:			
Subsample 2:			
MPN/100 L [3]**:**		**95% CONFIDENCE LIMITS** **LOWER:** **UPPER:**	
COMMENTS:			
ANALYST:			

[1]Enter the Total Sample Volume times 0.965 if a coliphage sample is taken, times 0.9 if archiving is required, times 0.865 if a coliphage sample is taken and archiving is required or times 1 if a coliphage sample is not taken and archiving is not required.
[2]Must be at least 100 L for source water and 1000 L for finished water.
[3]Value calculated from the Quantitation of Total Culturable Virus form as described in the Virus Quantitation section of Part 3.

TOTAL CULTURABLE VIRUS DATA SHEET

SAMPLE #:

	Total Number of Replicates					
	Subsample 1			Subsample 2		
Sample	**Inoculated**	**Without CPE**	**With CPE**	**Inoculated**	**Without CPE**	**With CPE**
1st Passage Neg. Cont.						
Pos. Cont.						
Undiluted						
1:5 Dil.						
1:25 Dil.						
2nd Passage[1] Neg. Cont.						
Pos. Cont.						
Undiluted						
1:5 Dil.						
1:25 Dil.						
3rd Passage[2] Neg. Cont.						
Pos. Cont.						
Undiluted						
1:5 Dil.						
1:25 Dil.						

[1]A portion of medium from each 1st passage vessel, including controls, must be re-passaged for conformation. The terms "Undiluted," "1:5 Dilution" and "1:25 Dilution" under the 2nd and 3rd Passage headings refer to the original sample dilutions for the 1st passage. If higher dilutions are used, record the data from the three highest dilutions showing positive results and place the actual dilution amount in the sample column.

[2]Samples that were negative on the first passage and positive on the 2nd passage must be passaged a third time for conformation. If a third passage is required, all controls must be passaged again.

QUANTITATION OF TOTAL CULTURABLE VIRUS DATA SHEET

SAMPLE NUMBER:

Sample	Number Replicates inoculated	Number with CPE	MPN/mL[1]	95% Confidence Limits	
				Lower	Upper
Undiluted Samples					
Subsample 1					
Subsample 2					
Total Undiluted					
Subsample 2 results (Dilutions Required)					
Undiluted					
1:5 Dilution					
1:25 Dilution					

[1]**Use the values recorded in the Total Undiluted row to calculate the MPN/mL result and confidence limits when dilutions are not required. If dilutions are required, base the calculation upon the values recorded in the Undiluted, 1:5 Diluted and 1:25 Diluted rows for subsample 2. If higher dilutions are used for subsample 2, record the data from the three highest dilutions showing positive results and place the actual dilution amount in the sample column. The MPN/mL and 95% Confidence Limit values must be obtained using the computer program supplied by the U.S. EPA.**